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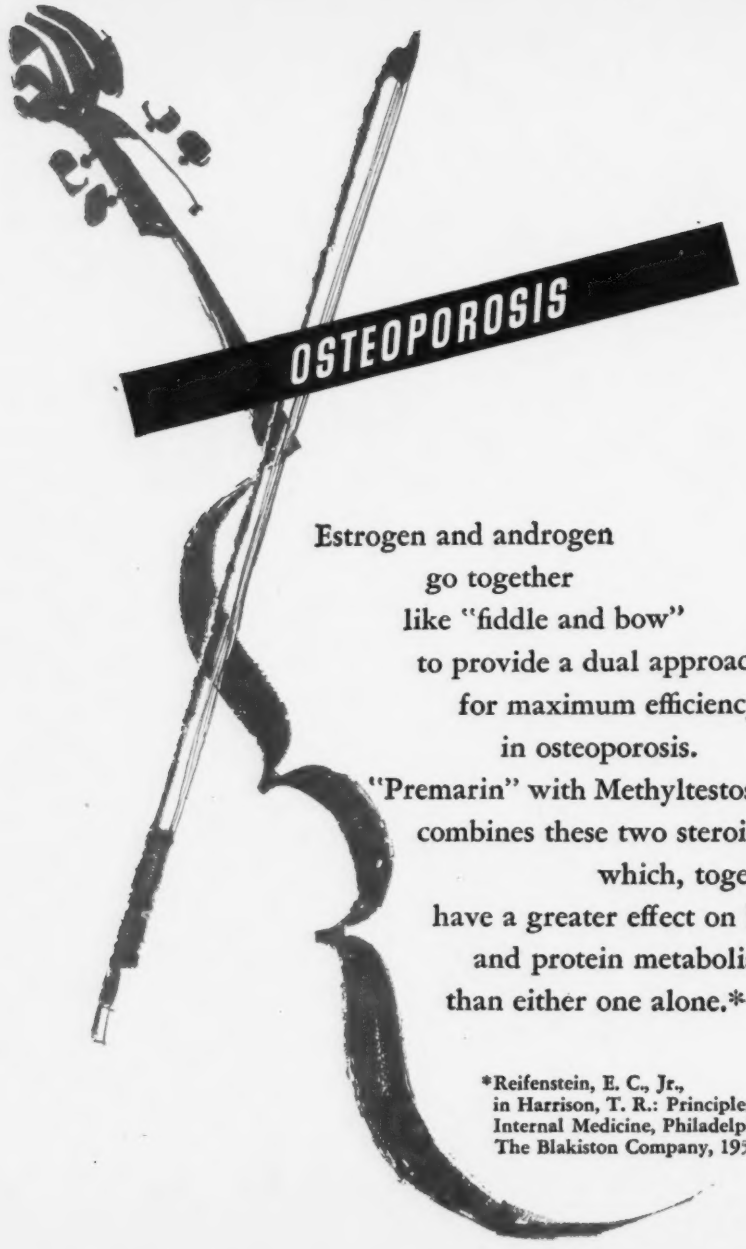

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ENDOCRINOLOGY

VOLUME 53 JULY, 1953 NUMBER 1

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NEUROGENIC EFFECTS OF UTERINE DISTENTION ON THE ESTROUS CYCLE OF THE EWE[1]

WARD W. MOORE[2] AND ANDREW V. NALBANDOV

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THE role of the uterus and its innervation in the regulation of cyclic reproductive phenomena and in the maintenance of corpora lutea has been little studied. Dickinson and Smith (1913) showed that local distention of the cervical portion of the uterus stimulated the ovaries of women with involuted and infantile uteri. Selye (1934) was able to maintain corpora lutea of pregnancy and prolong "gestation" in rats by substituting paraffin for the products of conception. The paraffin was said to stimulate the anterior pituitary gland causing persistence and function of the luteal tissue. The link between the stimulus arising in the uterus and the adenohypophysis was assumed to be neural. Attempts by Bradbury (1941) and Greene (1941) to confirm these results failed.

Apparently the ovaries and uterus can function normally without depending upon their respective nerve supplies. Denervation of the ovaries has no effect upon ovarian function, for transplanted ovaries function in a normal manner. Reynolds (1949) states that implantation, pregnancy, and parturition can proceed normally in the denervated uterus.

Harris (1948) and Sawyer, Everett, and Markee (1950) have shown that a neuro-humoral mechanism, operating between the hypothalamus and the adenohypophysis, is involved in the release of ovulating hormone (LH) in

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[1] We wish to thank Dr. Upson S. Garrigus for his cooperation in providing the animals used, Dr. Horace W. Norton for his aid in the statistical analysis of the data, and Dr. James A. Welch for supplying ovarian and uterine histological preparations which were taken from normal ewes throughout the estrous cycle.

[2] Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Physiology in the Graduate College of the University of Illinois, Urbana, Illinois, June 1952, under the direction of Drs. Andrew V. Nalbandov and Robert E. Johnson.

the rat and rabbit. Hansel and Trimberger (1951) have found that a similar mechanism is also present in the dairy cow.

The mechanism by which the corpus luteum is caused to persist following fertilization in mammals which ovulate spontaneously during each reproductive cycle and which do not show pseudopregnancy following cervical stimulation is not known. Similarly, the cause of the regression of the corpus luteum is not known. The role of the afferent nerves of the cornua of the uterus in establishing and maintaining pregnancy is also unknown.

Experiments were designed to determine whether uterine distention similar to that which may be caused by an implanting blastocyst during the early luteal phase of the estrous cycle of the ewe would cause prolongation of the life of the corpus luteum. The ewe has an estrous cycle lasting about 16 days, ovulates spontaneously and does not become pseudopregnant (McKenzie and Terrill, 1937).

MATERIALS AND METHODS

All the sheep used were about 1 year old at the start of the experiment. They were permitted to complete at least one normal estrous cycle before they were placed on experiment. Sixty-three ewes were checked for heat twice daily with a vasectomized ram according to the procedures described by McKenzie and Terrill (1937). Surgery was performed under Nembutal anesthesia three days after the first signs of heat. Both ovaries were examined for the presence of fresh ovulations and old corpora lutea. The size and number of follicles was recorded.

In the initial stages of this work, beads made of Fisher Tissuemat (M.P. 56–58° C.), about 10 mm. in diameter, were attached to surgical sutures, inserted through an incision into the lumen of one uterine cornu and fastened to the uterine wall of six ewes. However, the paraffin was softened by body heat and was dislodged from the suture. Hence plastic beads of unknown chemical composition were substituted for the paraffin in all subsequent experiments.

The results indicated that uterine distention modified the normal function of the anterior pituitary gland. To learn whether this uterus-pituitary interrelationship was neural or humoral in origin, a surgical operation was devised in which that portion of the uterine cornu containing the bead was denervated. This was accomplished by completely severing a segment of the cornu, about 3 cm. in length, from the rest of the uterus and from all supporting structures. The resected part was then sutured to the cut surfaces in order to assure reestablishment of the circulation to the resected portion. The procedure used is shown in Figure 1, where A, B, and C show the lines of incision and suturing. The typical appearance of a resected portion of the cornu is shown on the opposite cornu of the uterus, where each white arrow points to a healed incision.

Exploratory experiments were conducted on 2 ewes to ascertain the rate and degree of revascularization of a resected segment after using this surgical procedure. Both ewes were laparotomized two days after resection, when it was found that healing had occurred normally and that the resected portion had become revascularized. Because the segment was morphologically indistinguishable from the rest of the uterus, it is probable that revascularization had begun shortly after the segment had been reunited with the rest of the uterus.

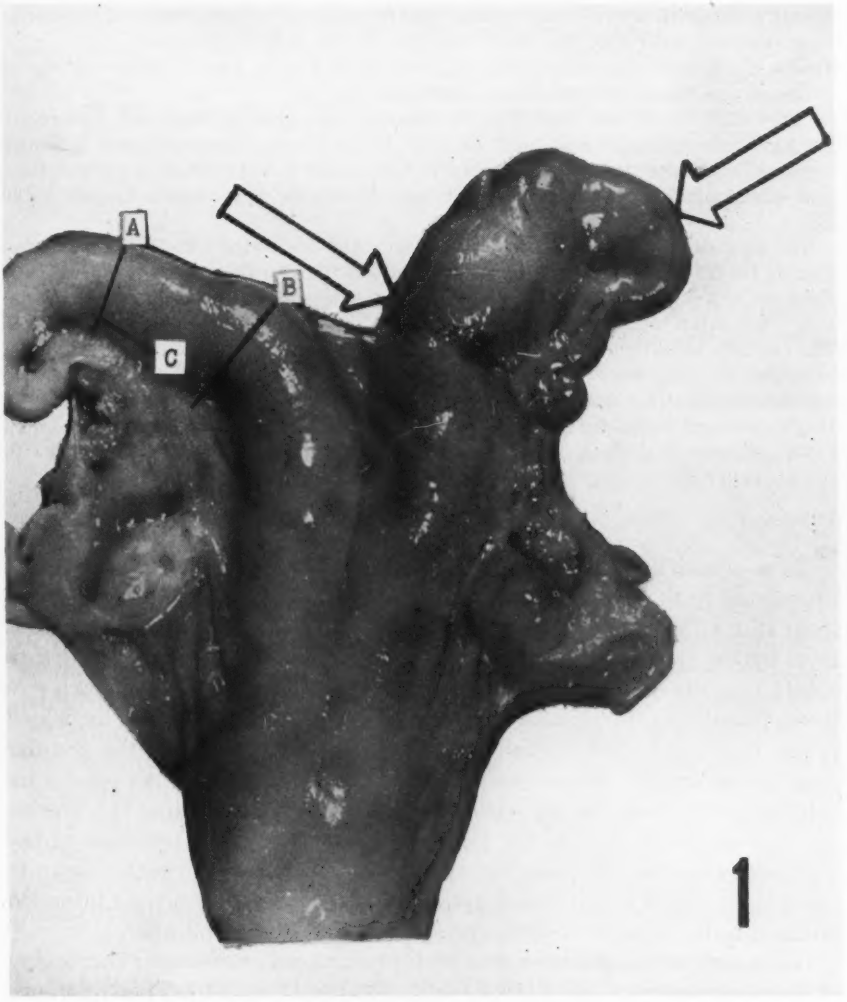


FIG. 1. Procedure employed in denervating a section of the uterine cornu of the ewe. Description in text.

Fifty yearling Western ewes were divided at random into 5 equal groups.

Group A consisted of ewes in each of which a plastic bead, 11 mm. in diameter, was sutured into the lumen of one of the uterine cornua.

Group B consisted of ewes in each of which a plastic bead, 8 mm. in diameter, was sutured into the lumen of one of the uterine cornua.

Group C consisted of ewes which were operated in the same manner as those of Groups A and B, but no bead was inserted.

Group D consisted of ewes in which a segment (30–35 mm. in length) of one of the uterine cornua was denervated and either an 8 or an 11 mm. bead was inserted.

Group E consisted of ewes in which a segment (30–35 mm. in length) of one of the uterine cornua was denervated, but no bead was inserted.

Only 46 of the 50 ewes showed normal estrous cycles prior to treatment. As a result, some groups consisted of nine ewes (Table 2). In the course of the experiment it became apparent that some treatments did not affect the cycles over prolonged periods of time, and seven animals from Groups C and E were transferred at random to Groups A and B.

The animals were killed at random after they had completed 1 to 13 estrous cycles. During the terminal cycle of each, the ewe mated with a fertile ram. The interval from the onset of the last estrus to the time of autopsy varied from two to fifty days. At autopsy, the entire reproductive tract was removed intact, photographed, and described. The oviducts and uterine cornua were flushed with physiological saline solution in order to recover ova. This was not done when pregnancy was obvious and in four animals which had entered anestrus. The ovaries and representative sections of the uteri were fixed in Bouin's solution, imbedded in paraffin, sectioned at eight microns, and stained according to the procedure of Milligan (1946) for histological examination. The data concerned with the length of the estrous cycle were analyzed statistically.

RESULTS

Ewes in which paraffin beads were used to distend the uterus were found to respond to the treatment by a decrease in the length of the estrous cycle from 16.1 ± 0.91 to 12.9 ± 5.13 days. The difference is significant at the 5% level by the Behrens-Fisher test (Fisher, 1939). The cycles also tended to return to normal lengths after two or three cycles. At autopsy of each ewe it was found that the paraffin bead had been torn from the thread by which it was fastened to the uterine wall, and had lodged between the annular rings of the cervix. It was inferred that the shortened estrous cycles coincided with the time during which the paraffin was distending the uterine cornu, and that as soon as the paraffin became detached, terminating the uterine distention, the animals resumed normal cycles. To test this assumption plastic beads which would not be easily dislodged from their intended position in the uterine cornua were substituted for the paraffin.

Distention of the uterine cornu with plastic beads caused the shortening of the normal estrous cycle (16.1 ± 0.91 days) to 11.7 ± 2.07. This difference is significant at the 0.1% level (Fisher, 1939).

To test the original assumption that uterine distention led to shortening of the cycle, while termination of distention permitted return to the normal cycle length, plastic beads were placed in the uterine lumen and were later removed. While the beads were present in the uterus, the interval between estrous periods was reduced to 8.2 days; after removal of the beads the normal cycle length (16.1 days) was resumed (Table 1).

From these data it was inferred that mechanical stimulation of the

TABLE 1. COMPARISON ON THE SAME ANIMALS OF THE EFFECT OF THE PRESENCE OR ABSENCE OF UTERINE DISTENTION ON THEIR ESTROUS CYCLES

Ewe	Length of first cycle (days)	Cycle lengths after uterine distention (days)	Cycle lengths after removal of distention (days)
913	16	10, 5, 5, 5, 9	15, 17, 15
378	16	6, 15, 5	16, 16, 18
858	17	6, 14, 7	15, 16, 17
Mean	16.3	8.2	16.1

sensory nerve endings of the uterine cornua initiates events which induce a change from the normal secretion of hypophyseal gonadotrophins which, in turn, causes the reproductive cycle to be altered. To test this assumption, experiments were undertaken in which portions of the cornu containing the bead were denervated in the manner described.

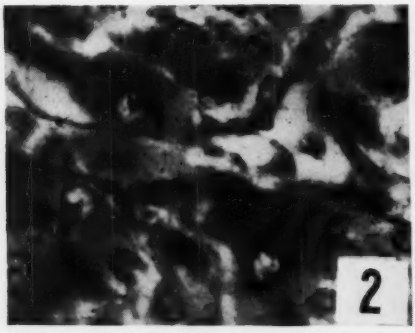
The results of the experiment show that uterine distention in the intact ewe causes a significant ($P = 0.001$) shortening of the estrous cycle of the ewe (Table 2). However, if the section of the uterus which contained the bead was denervated, uterine distention produced by a plastic bead had no effect upon the length of the estrous cycle. Similarly, denervating a section of the uterus without inserting a bead had no effect upon the length of the estrous cycle. Also, the sham operation did not alter the length of the estrous cycle. Statistical analysis showed that there is no significant difference between sheep, nor between cycle-orders in the average length of the cycles within experimental groups. However, it should be noted that uterine distention and denervation of a section of the uterus increased the variability of the cycle lengths.

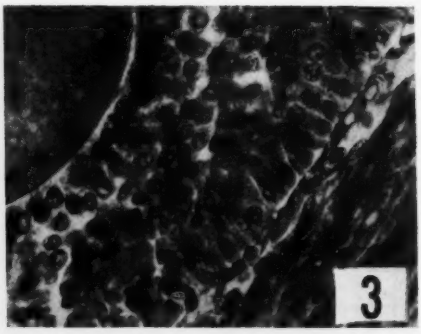
To learn whether ovulation accompanied heat in animals into which beads had been inserted but which had normal uterine innervation, and whether the ova were fertilizable (if ovulation did occur), the animals were killed at various times after the onset of heat and the oviducts were flushed

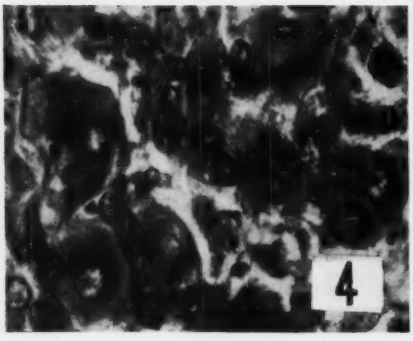
TABLE 2. EVIDENCE OF A NEUROGENIC EFFECT OF UTERINE DISTENTION ON THE ESTROUS CYCLE ON THE EWE

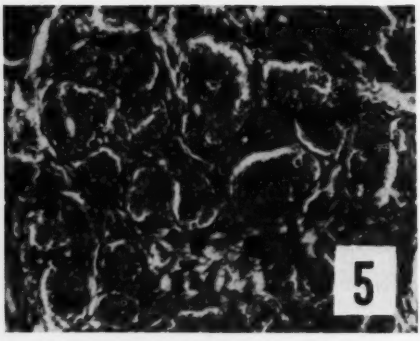
Treatment	Number of ewes	Number of cycles	Mean cycle length (days)
11 mm. bead	14	60	11.8 ± 0.94[1]
8 mm. bead	12	58	12.0 ± 1.11
Sham operated	9	35	16.4 ± 0.20
Denervated + bead	9	22	16.1 ± 0.50
Denervated	9	34	16.2 ± 0.38
Controls[2]	46	46	16.3 ± 0.19

[1] Standard error of the mean.
[2] Refers to the length of the first cycle of all of the 46 ewes in the experiment.









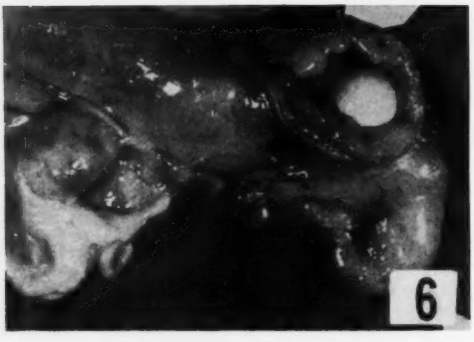


FIG. 2. Luteinized ovarian follicle wall of a ewe whose intact uterus had been distended with a plastic bead. Many functional luteal cells are present. ×690.

FIG. 3. Section showing slight luteinization of the theca folliculi from a ewe on the 35th day of pregnancy. ×460.

FIG. 4. Corpus luteum of pregnancy taken from same ewe whose follicle is pictured in Figure 3. ×690.

FIG. 5. Section of the endometrium through the denervated portion of the uterus of a

for ova. In 18 of 22 ewes it was found that heat was accompanied by one or more ovulations, and ova or embryos were recovered in 17 of the 18. Of the 17, nine were fertilized and three of those were implanted, showing that such ova can be fertilized and implanted. The implantation rate may be actually higher, for many of these animals were killed too early to give good opportunity for implantation. None of the experimental procedures described interfered with ovulation and implantation. However, we doubt whether pregnancy can run its normal course in animals whose uteri have been partially denervated by the method employed in this study. During the later stages of pregnancy observed in this study (up to 50 days) the fetuses were dead and in the process of being resorbed.

Two generalizations can be made concerning the effects of uterine distention on ovarian and endometrial histology. 1) Luteinization of tertiary follicles occurs after distention of the intact uterus, but does not follow distention of a denervated portion of the uterus, except during pregnancy (Figs. 2, 3, 4). 2) The endometrium reaches a peak in secretory function more quickly after corpus luteum formation following distention of the intact uterus than either in normally cycling ewes, or in ewes having a portion of the uterus denervated (Table 3).

It is important to emphasize that segments of the uterus which were denervated in the manner described (Figs. 1 and 6) revascularized satisfactorily and did not become necrotic, except in one case in which a slight degree of necrosis was found (Fig. 5). The endometrium in the resected portions was fully functional by the time of autopsy and did not differ histologically from the endometrium of the remainder of the uterus. No attempt was made to learn whether nerves regenerated in these sections. The presence of a direct neural connection between the uterus and the ovaries as a possible route of action on the estrous cycle has not been ruled out. However, neither direct nor indirect axonal connection between the uterus and the ovaries has ever been demonstrated.

DISCUSSION

It is well established that a neurohumoral link exists between the uterine cervix and the anterior pituitary gland. Such a connection between the

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ewe. Slight degree of necrosis is present, but this portion of the uterus is functional. 37 days after denervation. Section is taken from the uterine cornu near the bead shown in Figure 6. ×180.

FIG. 6. Uterine cornu from a ewe in Group D. A section of the uterus had been denervated and the plastic bead is in place. The three lines of incision can barely be seen. Histological examination showed a secretory endometrium with a slight amount of necrosis, ×1. See Figure 5.

TABLE 3. EFFECTS OF DISTENDING THE INTACT UTERUS OR A DENERVATED SECTION OF THE UTERUS OF THE EWE ON THE OVARIAN AND UTERINE HISTOLOGY

Ewe number	Group[1]	Day of cycle killed	Condition of follicles	Estimated age of corpus luteum	Condition[2] of endometrium	Presence of a new ovulation
721	A	5	luteinized	5	9	yes
709	A	5	luteinized	5	11	yes
723	A	(x)	luteinized	7		no
741	A	13	luteinized	7	11	yes
665	A	8	luteinized	13	9	yes
657	A	3	luteinized	4	11	yes
698	A	9	luteinized	7	11	yes
696	A	5	luteinized	20	9	no
739	A	15(x)	normal	13	9	yes
738	A	2	normal	3	4	yes
743	A	4	normal	5	5	yes
672	A	2	normal	2	4	yes
693	B	12(x)	luteinized	9	9	yes
644	B	1	luteinized	9	5	no
619	B	4	luteinized	4	11	yes
634	B	3	luteinized	1	7	yes
761	B	(y)	luteinized	2	4	yes
705	B	4	luteinized	19	11	no
666	B	3	luteinized	3	9	yes
724	B	3	luteinized	3	7	yes
629	B	3	luteinized	7	9	no
673	B	3	luteinized	4	11	yes
678	D	(x)	luteinized	9		no
689	D	3	normal	3	3	yes
631	D	2	normal	2	4	yes
731	D	2	normal	1	16	yes
622	D	3	normal	3	5	yes
630	D	7	normal	9	5	yes
753	D	(x)	luteinized	9		no
650	D	5	normal	5	5	yes
702	D	(x)	luteinized	9		no

[1] Four animals were considered anestrus and were not included in this study.
[2] Estimation of the day of the cycle which the endometrium best represents.
(x) Pregnant.
(y) Had not been in heat for 62 days.

uterine cornua or uterine body and the pituitary gland has been suspected, but has never been shown to exist. The original assumption under which this work was undertaken turned out to be incorrect. Uterine distention of the type used did not cause the corpus luteum to persist. However, it was found that the uterine innervation does play a role in the regulation of the secretion of anterior pituitary gonadotrophins. How this observation fits into normal reproductive phenomena remains to be ascertained. Whether lesser degrees of distention will modify the results reported here is being studied at the present time.

It is important to note that the results observed are of neural rather than of humoral origin. This is shown by the fact that the shortening of the cycles occurred only when a bead was present in an intact uterus, while this phenomenon disappeared either if the bead was removed or if the uterine

segment containing the bead was denervated. In the initial experiment in which the paraffin used for distention eventually became lodged in the cervix, the resulting cervical distention did not prevent cycles of normal length. This further shows that termination of distention of the uterus causes the return of normal cycles.

Astwood and Greep (1938) state, that the uterus itself, the maternal placenta or the "metrial gland" are not concerned in this mechanism, e.g., luteal stimulation, is indicated by the fact that the presence of numerous large deciduomata in the uterus fail to prolong the length of pseudopregnancy, and any influence of uterine distention or of uterine hormones on luteal function is thus ruled out. However, Ershoff and Deuel (1943) were able to prolong pseudopregnancy in the rat with deciduomata. Their work was confirmed by Peckham and Greene (1948), who state that the results fail to indicate that prolongation of pseudopregnancy is an effect of deciduomata *per se*. It appears that this effect may be initiated by the physical distention of the uterus by deciduomata.

The nature of the endocrine changes produced by uterine distention is still being investigated. However, histological studies of the ovaries and endometria yield information on which several working hypotheses can be based. The accelerated rate at which follicles are matured and ovulated (in some instances as frequently as 4 to 5 days), suggests several possible explanations:

1) The control mechanism which normally participates in secretion or release of total pituitary gonadotrophins is altered by the continuous stimulation of the nerves of the uterine cornua.

2) The neurogenic stimulus from the distended uterine cornu increases the rate of FSH production and secretion, leading to a more rapid maturation of ovarian follicles.

3) The neurogenic stimulus leaves the rate of FSH secretion unaltered, but increases LH secretion.

That uterine stimulation (injury?) may selectively alter the rate of gonadotrophic hormone secretion, has been demonstrated for another species (chicken) by Huston and Nalbandov (1953). The data at hand do not indicate which of the three suggested explanations operate in sheep.

In addition to the probable changes produced in the rates of secretion of FSH and LH, uterine distention may cause an increased secretion of luteotrophic hormone. It was found that the ovulated follicles formed normal and mature corpora lutea.

In some of the ewes which ovulated at 4–6 day intervals one would expect to find several corpora lutea in various stages of development. Since this was not observed at autopsy, it is possible that corpora resulting from these ovulations degenerate more rapidly than do corpora formed in nor-

mal estrous cycles. Bunde and Greep (1936) and Greep (1938) have shown that the luteinizing fraction of the anterior pituitary does cause rapid involution of the persistent corpora lutea which follow hypophysectomy in the rat. The concept that LH is luteolytic may be used to explain the rapid involution of corpora lutea of ewes which had short cycles. In view of the increased rate of ovulation, there must have been increased LH secretion by the anterior pituitary. Additional information on this observation is being accumulated at the present time. Ovaries of animals with distended uteri also contained several heavily luteinized unovulated tertiary follicles. These and the corpora lutea presumably were the source of the progestin responsible for the pronounced progestational proliferation of the endometrium. Luteotrophic hormone (prolactin) has been shown to cause marked luteinization of unovulated tertiary follicles in the ewe (Moore, 1952). Thus, it is very likely that uterine distention causes an increased production of hypophyseal luteotrophin, in addition to altering the rates of FSH and LH secretion.

Studies are now being undertaken to ascertain the effect of distention of the uterus of the ewe with beads about one mm. in diameter. According to Green and Winters (1945) the diameter of the fertilized egg in the ewe at the time of implantation (8–9 days after ovulation) is from 0.7 to 1.0 mm.

The participation of the uterus and its innervation in hypophyseal and ovarian physiology has gone unnoticed. This study indicates that the uterus, through its afferent endings, has a regulatory effect on the events which make up the estrous cycle of the ewe. Although this phenomenon has been shown in only one species, similar regulation by the uterus on the pituitary-ovary axis may exist in other spontaneously ovulating species.

SUMMARY

1) Distention of the uterine cornu of the cycling ewe under the hormonal conditions present during the early luteal phase of the reproductive cycle, alters the endocrine activity of the pituitary-ovary axis and causes a significant shortening of the estrous cycle from 16.3 to 11.9 days.

2) The maintenance of normal cycles (16.1 days) in ewes following distention of a denervated segment of the uterus, indicates that this phenomenon is mediated over nervous pathways. Denervation alone does not affect the length of the estrous cycle (16.2 days).

3) Evidence is presented which indicates that following uterine distention there may be an increase in the secretion of FSH, LH and luteotrophin. Possible mechanisms of these changes are discussed.

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THE RESPONSE OF THE ADRENAL GLAND OF NORMAL AND ALLOXAN-TREATED PREGNANT RATS TO COLD EXPOSURE[1]

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MODIFICATION of human adrenal function by diabetes has been documented by reports of McArthur, Sprague and Mason (1950) and Hinkle, Conger and Wolfe (1950). Shepherd, Smith and Longwell (1952) found that alloxan-induced hyperglycemia did not significantly modify the response of the rat adrenal to cold exposure, as determined by depletion of ascorbic acid and cholesterol concentrations in the gland. This latter method of evaluation of adrenal function has been applied to pregnant rats, both normal and alloxan-treated. Whereas the treatment with alloxan did not alter the reactions of the gland of the pregnant rat to a significant extent, the effect of pregnancy is quite evident in the lower concentrations of adrenal ascorbic acid and cholesterol, and in the low levels reached by these substances when the animal was exposed to cold. The results of the investigation are detailed below.

METHODS

Female, albino rats from our own colony were maintained on a commercial stock rat food preparation. At 90 to 150 days of age they were bred, and the establishment of pregnancy was ascertained by vaginal smear examination. After subjection to the experimental procedures described below, the pregnant animals were sacrificed on the 16th or 21st day of gestation. The gestation period of this strain of rats is 22 days. Nembutal was used to induce anesthesia, and the adrenal glands were removed for determination of ascorbic acid and cholesterol as a measure of the response to stress stimuli. Ascorbic acid was determined by the method of Roe and Keuther (1943) and cholesterol by the method of Schoenheimer and Sperry (1934) after extraction of the gland with acetone-alcohol. Nelson's (1944) method was applied to the determination of blood sugar.

Hyperglycemia was induced by the intravenous administration of alloxan, 50 mg. per kg. via the tail vein. This procedure was done 3 to 5 days prior to the 16th or 21st day of

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gestation, the time at which the stress stimulus was applied. The use of the term "diabetes" to describe the condition of these animals is herein avoided. Interference with normal metabolic reactions by alloxan had existed for a very short time. Diabetes, as it is usually understood, implies an abnormality of carbohydrate metabolism which has induced characteristic changes in the metabolism of fat and protein. These changes require time, the exact time being a variable which has not been examined in the individual rats. Too long an exposure to hyperglycemia was impractical in these experiments due to the danger of interruption of pregnancy. Therefore, these animals might perhaps be designated simply as animals with hyperglycemia induced by alloxan.

On the 16th or 21st day of gestation, the animals were subjected to the following experimental procedures: 1) normal animals kept at room temperature, no stress; 2) normal animals exposed to a temperature of 1–3° C. for 4 hours; 3) normal animals exposed at 1–3° C. for 1 hour; 4) alloxan-treated rats maintained at room temperature, no stress; 5) alloxan-treated animals exposed at 1–3° C. for 4 hours; 6) rats fasted for 24 hours and then exposed to 1–3° C. for 4 hours.

RESULTS

The values for adrenal ascorbic acid and cholesterol for all groups are recorded in Table 1. The ascorbic acid concentration[3] for the non-pregnant rats was 366 ± 6.6 mg./100 gm. as compared to concentrations for normal,

TABLE 1. THE EFFECT OF EXPOSURE TO COLD ON THE CHOLESTEROL AND ASCORBIC ACID CONCENTRATIONS OF THE ADRENAL GLANDS OF PREGNANT RATS

	Treatment	Number of rats		Ascorbic acid mg. per 100 gm.		Number of rats		Cholesterol mg. per 100 mg.	
		16th day	21st day	16th day	21st day	16th day	21st day	16th day	21st day
Normal	Normal No Stress	10	5	275 ± 12.4	319 ± 12.5	11	11	1.19 ± .08	2.22 ± .10
Normal	Cold Stress[1] 4 hours	7	5	228 ± 16.8	209 ± 15.3	7	5	0.88 ± .13	0.885 ± .17
Normal	Cold Stress 1 hour	7	7	263 ± 12.6	245 ± 12.4	7	7	1.38 ± .08	1.76 ± .38
Alloxan-treated	Alloxan-treated,[2] No Stress	5	7	313 ± 15.0	345 ± 15.8	5	5	2.18 ± .40	1.32 ± .10
Alloxan-treated	Alloxan-treated, Cold Stress 4 hours	6	5	246 ± 36.0	239 ± 30.0	6	6	0.65 ± .12	1.02 ± .15
Fasted	Normal Pregnant, Fasted, Cold Stress, 4 hours	6	7	234 ± 33.0	183 ± 17.8	5	5	0.54 ± .06	0.74 ± .05

[1] Cold stress animals were subjected to a temperature of 1–3° C.
[2] Alloxan-treated animals received 50 mg. per kg. via a tail vein 3–5 days prior to the cold exposure.

pregnant rats at room temperature of 275 ± 12.4 (16th day of gestation) and 319 ± 12.5 (21st day of gestation) mg./100 gm. Thus normal pregnancy alone is seen to cause a reduction of adrenal ascorbic acid. Exposure of the animal to 1–3° C. for 4 hours caused a further reduction on the 16th and

[3] All values expressed as the mean and standard error of the mean.

21st gestation days to 228 ± 16.8 and 209 ± 15.3 mg./100 gm., respectively. Reduction to values of 263 ± 12.6 (16th day) and 245 ± 12.4 (21st day) mg./100 gm. was caused by exposure to cold for 1 hour.

After treatment with alloxan the adrenal ascorbic acid values were 313 ± 15.0 mg./100 gm. on the 16th day of gestation and 345 ± 15.8 mg./100 gm. on the 21st day in animals maintained at room temperature. The values in comparable groups after exposure to cold were 246 ± 36.0 and 239 ± 30.0 mg./100 gm. The rats which had received alloxan had blood sugar values between 178 and 598 mg.% at the time they were sacrificed.

The cholesterol concentration of the adrenals of 13 normal, non-pregnant rats maintained at room temperature was $3.22 \pm .17$ mg./100 mg., whereas pregnant rats, normal and not stressed, had a concentration of $1.19 \pm .08$ mg./100 mg. on the 16th day of gestation and $2.22 \pm .10$ mg./100 mg. on the 21st day. Exposure to cold resulted in a reduction of these values to $0.88 \pm .13$ mg./100 mg. on the 16th day of gestation and to $0.885 \pm .17$ mg./100 mg. on the 21st day. No noteworthy change was noted after only one hour in the cold either on the 16th or 21st day, the values being $1.38 \pm .08$ and $1.76 \pm .38$ mg./100 mg., respectively. After treatment with alloxan the cholesterol concentrations at room temperature were $2.18 \pm .40$ mg./100 mg. on the 16th day of gestation and $1.32 \pm .10$ mg./100 mg. on the 21st day. In alloxan-treated animals exposure to cold again caused a reduction in concentration to $0.65 \pm .12$ mg./100 mg. on the 16th day. A less marked response was obtained on the 21st day, on which the mean value was $1.02 \pm .15$ mg./100 mg.

None of the animals in the experiments reported above was fasted before being subjected to the stress stimulus. In spite of this there was a reduction of both ascorbic acid and cholesterol in the adrenal. Shepherd, Smith and Longwell (1952) reported that fasting of the normal, non-pregnant rat was an important part of the experimental preparation if cold as a stress stimulus were to produce marked depletion of adrenal cholesterol. Therefore, pregnant animals were fasted 24 hours prior to cold exposure, and the lowest cholesterol concentrations observed, except for the alloxan-treated animals on the 16th day, were seen in these rats, $0.54 \pm .06$ mg./100 mg. and $0.74 \pm .05$ mg./100 mg. on the 16th and 21st days, respectively.

DISCUSSION

Pregnancy so alters metabolic balance or endocrine balance that the value for adrenal cholesterol is significantly lower than in the non-pregnant animal. This fact has been reported previously by Anderson and Sperry (1937), who reported adrenal cholesterol values of 1.62 mg./100 mg. on the 18th day of gestation compared to a maximum value of 2.84 mg./100 mg.

for the non-pregnant rat. A similar reduction in the pregnant rabbit was observed by Chamberlain (1930). Whether this can be interpreted as evidence that pregnancy consitutes a "stress stimulus" is not determined by this type of experiment. However, a modified resting level does exist, and if this is a reflection of increased adrenal activity (Venning, 1946), the gland must function at an equilibrium level of cholesterol which differs markedly from that of the non-pregnant rat, since it is able further to respond by a decrease of cholesterol when an additional stress stimulus is applied. Similar observations were described by Jones, Lloyd and Wyatt (1951).

Numerous suggestions have been made concerning the existence in females of extra-adrenal sources of substances with adrenal cortical action. Cowie (1949) recorded for rats a longer survival time for females than for males after adrenalectomy, an advantage which was attributed to progesterone action. Pregnancy confers on dogs a prolonged survival time after adrenalectomy (Rogoff and Stewart, 1926). Furthermore, Gaunt and Hays (1938) were able to maintain adrenalectomized ferrets without supportive treatment during pseudopregnancy. Jailer and Knowlton (1950) suggested the placenta as the source of increased excretion of 17-ketosteroids and neutral reducing lipids by a pregnant patient with Addison's disease. The recent demonstration of adrenal cortical hormone activity in both human and equine placenta by Johnson and Haines (1952) gives support to the concept that adrenal cortical hormone activity during pregnancy may come from extra-adrenal sources in addition to the adrenal cortex. Thus, the low adrenal cholesterol of pregnant rats may be related in part to the physiological stress of pregnancy, and in part to a modification of endocrine balance brought about by sources of cortical hormone activity other than the adrenal.

Whereas the concentration of adrenal cholesterol in the non-stressed groups was apparently altered by the treatment with alloxan, the ability of the gland to respond to cold exposure is still evident. Indeed, in both the hyperglycemic and the normal animal the concentrations reached lower values than any we have ever observed in the non-pregnant animal. In normal, pregnant rats, as in the case of normal males, it appears that fasting, preliminary to the cold exposure, results in a more marked depletion of adrenal cholesterol.

The resting concentrations of adrenal ascorbic acid were lower in the pregnant animal than in the non-pregnant one. This finding is not in agreement with those reported by Lloyd (1952). The reason for this difference is not apparent. This substance responded to cold exposure by depletion in amount. As was found to be the case in non-pregnant animals, alloxan-

induced hyperglycemia did not modify the reactions of the adrenal in a manner which could be interpreted as evidence of interference with its normal function.

SUMMARY

Pregnant, albino rats, both normal and alloxan-treated, were subjected to cold exposure. The adrenal response was measured by changes of cholesterol and ascorbic acid concentrations. Both cholesterol and ascorbic acid were found to be lower in the normal, pregnant rat than in the non-pregnant female. Cold exposure induced a further decrease in both of these substances. Treatment of the pregnant rats with alloxan did not interfere with the decrease of concentration of adrenal cholesterol or ascorbic acid when the animals were exposed to cold.

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CAN THE OVARIAN HORMONES INHIBIT THE MAMMARY RESPONSE TO PROLACTIN?[1]

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THE question as to why lactation is not normally initiated during pregnancy still remains unanswered. The concept which has probably gained widest acceptance is that the ovarian hormones, particularly estrogens, inhibit the secretion of prolactin by the pituitary (Nelson, 1936). This view is held despite the fact that no direct evidence has yet demonstrated that the ovarian hormones can reduce prolactin either in the pituitary or circulation. On the contrary such studies have consistently shown that the ovarian hormones, particularly estrogens, increase the prolactin content of the pituitary (Meites and Turner, 1948a,b) and blood (Meites and Turner, 1942). This has led us to re-examine the possibility that the ovarian hormones may suppress lactation by rendering the mammary gland refractory to prolactin stimulation. Nelson (1936) first suggested that estrogens may inhibit the mammary response to prolactin, but the experimental data bearing on this have been meager and inconclusive and their validity have been challenged (Folley and Malpress, 1948).

EXPERIMENTAL

A total of 63 young, previously unmated, New Zealand White rabbits of both sexes were used in three experimental trials. The animals weighed from 5 to 7 pounds each at the beginning of the experiments. Intact males were used in the first experiment, orchidectomized males in the second experiment and ovariectomized females in the third experiment. A period of ten days was permitted to elapse after castration in order to remove the influence of the gonads. The animals were fed commercial rabbit pellets, and both food and water were available at all times.

All rabbits received daily subcutaneous injections for the first 25 days

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of 960 I.U. of estrone[4] and 1 I.U. of progesterone[5] in separate 0.1 cc. volumes of corn oil. Scharf and Lyons (1941) had previously shown that this combination elicited optimal mammary growth in male rabbits. From the 25th to 35th day, all except three control rabbits received 2 mg. daily of prolactin[6] by subcutaneous injection in 0.1 cc. of distilled water, together with one or both of the steroid hormones.

In order to determine whether the mammary glands were in a state of active growth during the last 10 days of each experiment, several rabbits

TABLE 1. LACTATIONAL RESPONSES IN RABBITS RECEIVING PROLACTIN WITH OR WITHOUT ESTRONE AND/OR PROGESTERONE

Exp.	Group and No. of rabbits	Treatment		Av. lactational rating per gland
		1st to 25th day	25th to 35th day	
		Intact male rabbits		
1	1 (3)	Est.[1]+Prog.[2]	—	0
	2 (5)	Est.[1]+Prog.[2]	Prolactin[3]+Est.+Prog.	less than 1
	3 (5)	Est.[1]+Prog.[2]	Prolactin[3]+Est.+Prog.	less than 1
	4 (5)	Est.[1]+Prog.[2]	Prolactin[3] only	3
	5 (5)	Est.[1]+Prog.[2]	Prolactin[3] only	3
		Castrate male rabbits		
2	1 (5)	Est.[1]+Prog.[2]	Prolactin+Est.+Prog.	less than 1
	2 (5)	Est.[1]+Prog.[2]	Prolactin only	3
	3 (5)	Est.[1]+Prog.[2]	Prolactin+Est.	2
	4 (5)	Est.[1]+Prog.[2]	Prolactin+Prog.	3
		Castrate female rabbits		
3	1 (5)	Est.[1]+Prog.[2]	Prolactin+Est.+Prog.	less than 1
	2 (5)	Est.[1]+Prog.[2]	Prolactin only	4
	3 (5)	Est.[1]+Prog.[2]	Prolactin+Est.	3
	4 (5)	Est.[1]+Prog.[2]	Prolactin+Prog.	4

[1] Est. = estrone, 960 I.U. daily.
[2] Prog. = progesterone, 1 I.U. daily.
[3] Prolactin, 2.0 mg. daily.

from each group were injected intravenously with 50 microcuries of radioactive phosphorus (P^{32}) per kg. of body weight, 4 hours prior to sacrifice. Radioautographs were prepared of individual mammary glands according to the technique outlined by Lundahl, Meites and Wolterink (1950)[7].

After killing the rabbits on the 36th day, the mammary glands were exposed and rated visually for intensity of lactation. Ratings of 0 to 4 were assigned each rabbit.

[4] and [5] The writers wish to express their appreciation to Dr. E. Gifford Upjohn of The Upjohn Co. for part of the crystalline estrone and progesterone used in this study.

[6] The highly purified prolactin was made available through the courtesy of Dr. R. W. Bates of E. R. Squibb and Sons, and was stated to contain 20–25 I.U. per mg.

[7] The writers are indebted to Dr. W. S. Lundahl of the Department of Natural Science, Michigan State College, for making these radioautographs.

RESULTS

The data for the 3 experiments are shown in Table 1. In the first experiment, in which intact males were used, the 3 control rabbits (group 1) which received both steroid hormones but no prolactin for the 35 days had no milk in their mammary glands. Groups 2 and 3 received both steroid hormones for the full 35 days and prolactin from the 25th to 35th day. No milk could be seen in their mammary glands although an occasional drop could be squeezed from one of the nipples. Groups 4 and 5 were injected with the two ovarian hormones for the first 25 days followed by prolactin alone for the next 10 days, and considerable lactation was evident

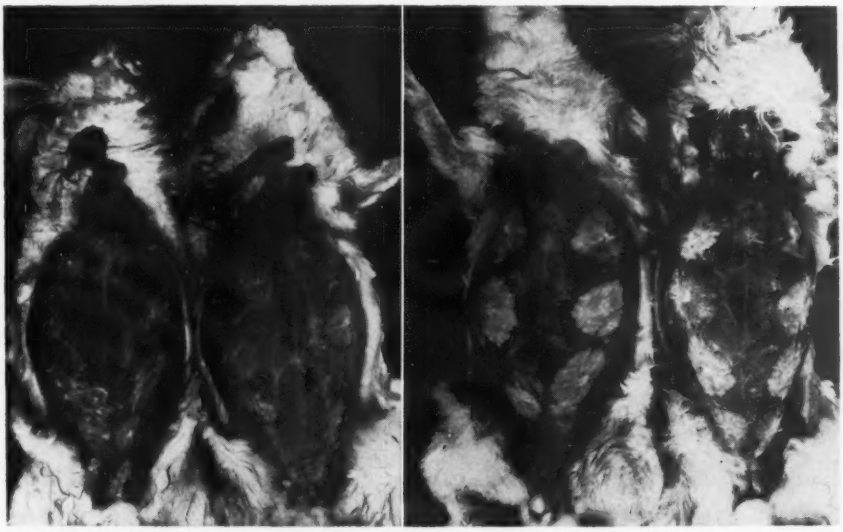

FIG. 1. Intact males. All 4 rabbits were injected with estrone and progesterone for the first 25 days. From the 25th to 35th day, the two rabbits on the left were injected with prolactin, estrone and progesterone, and show no milk in their mammary glands. The two rabbits on the right were injected with prolactin only during this period and show considerable lactation.

in all these rabbits (average rating of 3). The mammary glands of four representative rabbits from this experiment are shown in Figure 1.

The results of the 2nd experiment, performed on orchidectomized rabbits, indicate that groups 1 and 2 responded the same as the similarly treated intact male rabbits of the first experiment. Group 3, which received prolactin and estrone from the 25th to 35th days, had slightly less milk in their mammary glands than the rabbits injected with prolactin

alone (group 2). When progesterone was given together with prolactin during the last 10 days of the 35 day-experimental period (group 4), no inhibition of lactation was evident.

The data from the 3rd experiment, on ovariectomized rabbits, are essentially in agreement with those of the orchidectomized rabbits. However the degree of mammary growth and quantity of milk secreted were greater (Fig. 2) than in the male rabbits. Again estrone slightly inhibited prolactin

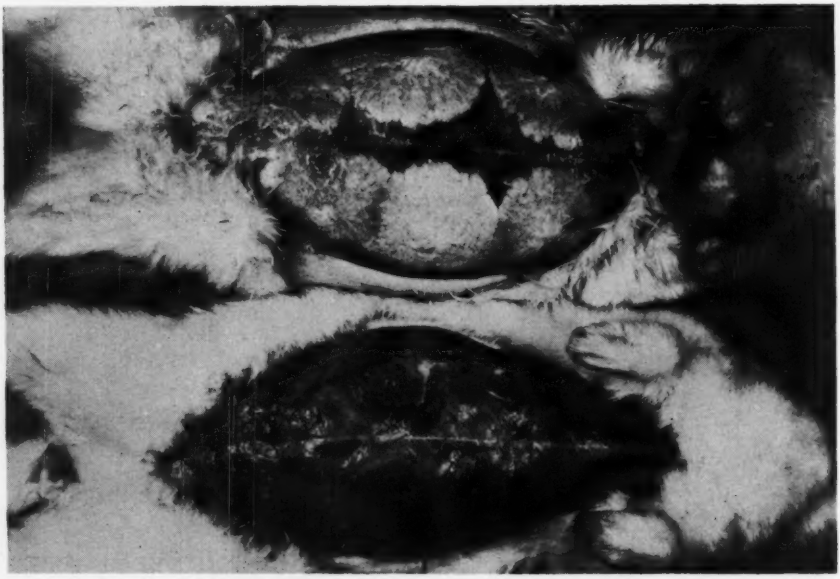

FIG. 2. Ovariectomized females. Both rabbits received estrone and progesterone for the first 25 days. From the 25th to 35th day, the rabbit above was injected with prolactin only and its mammary glands are filled with milk. The rabbit below received prolactin, estrone and progesterone, and shows complete inhibition of lactation.

action (group 3) while progesterone had no effect on lactation (group 4).

Radioautographs of mammary glands from three intact male rabbits of groups 2 and 4 of the 1st experiment are shown in Fig. 3. It can be seen that mammary growth in the two rabbits injected with both steroid hormones for the full 35 days was active and widespread, as indicated by the concentration of P^{32} in the peripheral and nipple areas (the regions of most active growth). The mammary gland of the rabbit from group 4, which received no steroid hormones from days 25 to 35, shows growth stasis as indicated by the more uniform distribution of P^{32} generally and a lesser concentration in the peripheral areas. The milk from this gland showed only a

minimal amount of radioactivity as compared to that found in the mammary tissue, an observation in accord with previous findings (Lundahl *et al.*, 1950). On the whole, these and the radioautographic data previously reported by us do not support the view of Lyons (1937, 1942) that prolactin stimulates mammary growth.

DISCUSSION

These results are believed to demonstrate that when estrone and progesterone are injected together in a ratio previously shown to promote optimal

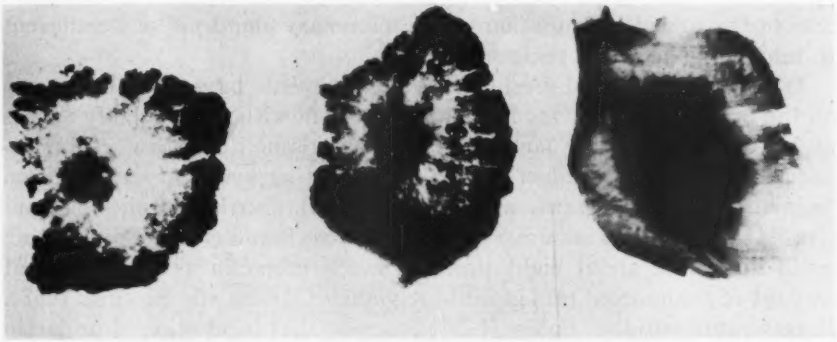

FIG. 3. From intact males. The two glands on the left are radioautographs from two rabbits given estrone, progesterone and prolactin from the 25th to 35th day. The gland on the right is from a rabbit given prolactin alone during this period. Active growth in the two glands on the left is indicated by the concentration of P^{32} in the peripheral and nipple areas. Growth stasis in the gland on the right is indicated by the more general distribution of P^{32}, with least in the peripheral areas. The milk from this gland showed but little radioactivity and did not appreciably influence the distribution of P^{32} in the mammary tissue.

mammary growth in male rabbits (Scharf and Lyons, 1941), the mammary tissue is not responsive to prolactin stimulation. When either was injected alone, progesterone produced no inhibition and estrone only a slight inhibition of the mammary response to prolactin. The dose of progesterone used, 1.0 mg. daily, can be considered physiological in the rabbit, since Corner and Allen (1930) demonstrated that the equivalent of 0.5 to 1.0 mg. daily was sufficient to maintain gestation following ovariectomy in this species. Even huge doses of progesterone have not depressed established lactation in rats (Folley, 1942) and women (Abarbanel, 1949), respectively. The 960 I.U. of estrone injected daily was probably above the physiological level, since Beerstecher (1942) reported that rabbits excrete a maximum of 180 rat units of estrogen per liter of urine during pregnancy.

Nelson (1937) reported that when estrogens and anterior pituitary ex-

tracts were injected into 4 hypophysectomized guinea pigs, lactation did not begin unless the estrogen injections were stopped. In another experiment however, Nelson (1934) found that lactation in parturient guinea pigs was not suppressed by estrogens if a sufficient dose of lactogenic extract was injected at the same time. Estrogens have shown little capacity to inhibit established lactation in ovariectomized as compared to intact rats (Anselmino and Hoffmann, 1936; Folley and Kon, 1938; Edelman and Gaunt, 1941), indicating that estrogenic stimulation of the ovaries to secrete progesterone probably occurs in the intact rats (Fauvet, 1941; Barsantini *et al.*, 1946; Masson, 1948). There is thus some question as to whether estrogenic stimulation of the mammary gland *per se* is sufficient to inhibit the action of prolactin.

The results of several recent *in vitro* experiments have a direct bearing on the present findings. Incubation of prolactin with various body tissues at 38° C. indicated that lactating mammary tissue may have greater capacity to inactivate prolactin than non-lactating mammary tissue from pregnant animals (Sgouris and Meites, 1952). Further studies demonstrated that lactating mammary tissue from rats four days after parturition could inactivate about eight times as much prolactin as an equivalent amount of tissue from rats in mid-pregnancy (Meites and Sgouris, 1953). In respiratory studies, Folley (1952) reported that incubation of prolactin with mammary tissue from lactating rats increased the rate of net gas evolution, whereas no change occurred when mammary tissue from pregnant rats was incubated with prolactin. These data further suggest that the action of the two ovarian hormones on the rat mammary gland during pregnancy render it refractory to prolactin stimulation.

SUMMARY

A total of 63 albino rabbits of both sexes, intact and castrate, were injected with 960 I.U. of estrone and 1 I.U. of progesterone daily for 25 days in order to induce optimal mammary development. From the 25th to 35th days, all except three rabbits were injected daily with 2 mg. of prolactin (20–25 I.U. per mg.), with or without one or both of the steroid hormones. At the end of the 35th day, the rabbits were killed and the mammary glands were exposed and rated for intensity of lactation. The extent of mammary growth induced by these treatments was determined by injecting radioactive phosphorus (P^{32}) 4 hours prior to sacrifice of the rabbits, and then making radioautographs of the mammary glands.

The rabbits given prolactin and both steroids during the last 10 days of the 35 day experimental period had practically no milk in their mammary glands (av. rating of less than 1); those injected with prolactin alone had mammary glands filled with milk (av. rating of 3 to 4); those given pro-

lactin and progesterone showed no inhibition in lactational response (av. rating of 3 to 4); and the rabbits injected with prolactin and estrone showed only a slight decrease in milk secretion (av. rating of 2 to 3). The radioautographs indicated the presence of intensive mammary growth in the rabbits treated with both steroids throughout the entire 35 day period but not in the rabbits given both steroids only for the first 25 days. It is concluded that in the doses employed, estrone and progesterone together can effectively inhibit the milk-secreting action of prolactin on the mammary gland of the rabbit, whereas progesterone alone is ineffective and estrone alone is only slightly effective in this respect.

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AN EXTRA-ADRENAL EFFECT OF SOME COMMERCIAL PREPARATIONS OF CORTICOTROPIN UPON THE WORK PERFORMANCE OF THE HYPOPHYSECTOMIZED RAT

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THE capacity of the rat to work in response to the faradic stimulation of muscle is rapidly lost following either adrenalectomy or hypophysectomy. Treatment of the hypophysectomized rat by the intermittent injection of either adrenal cortical extract (Ingle, 1938) or of corticotropin (Ingle, Moon and Evans, 1938) partially sustains the capacity for muscle work. More recently, we (Ingle, Nezamis and Morley, 1952) have shown that the continuous intravenous injection of a commercial preparation of corticotropin sustains the ability of the hypophysectomized rat to perform normal amounts of work.

The data of this report show that adrenal cortical extract only partially supports the ability of either the hypophysectomized or the hypophysectomized-adrenalectomized rat to work but that a combination of adrenal cortical extract with certain preparations of corticotropin fully sustains normal work performance of hypophysectomized rats in either the presence or absence of the adrenal glands.

Jacot and Selye (1952) have noted certain extra-adrenal morphologic effects of some preparations of corticotropin.

METHODS

Male rats of the Sprague-Dawley strain were obtained from the Upjohn breeding colony and maintained on Archer Dog Pellets until they reached a weight of 200 ± 2 gm. The work test was carried out according to Ingle (1944). The animals were anesthetized with phenobarbital sodium and cyclopal sodium and were subjected to the stimulation of muscle immediately following hypophysectomy or hypophysectomy-adrenalectomy. The gastrocnemius muscle of the left hind leg was weighted with 100 gm. A Nerve Stimulator, Model B (Upjohn) was used to deliver 5 pulses per second. The duration of each pulse was 20 milliseconds and the intensity was 20 milliamperes. The distance the weight was lifted was recorded on automatic work adders. Each recorder revolution represented approximately 400 gram-centimeters of work. Stimulation was continued until death or for 48 hours. The animals were enclosed in a cabinet with temperature constant at $28 \pm 0.5°$ C.

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Three commercial preparations of corticotropin (ACTHAR, Armour; ACTH, Wilson; and ACTH, Upjohn) derived from the pituitary glands of domestic packing house animals were used. The potency is expressed in terms of U.S.P. units as determined by the adrenal ascorbic acid depletion test (Sayers, Sayers and Woodbury, 1948). Each preparation was found to be stable at room temperature. The corticotropin was added to physiological saline together with penicillin G sodium (5000 units per 20 ml.) with and without adrenal cortical extract. Adrenal cortical extract (Upjohn) and hydrocortisone were each given without corticotropin in some experiments. The adrenal cortical extract represented the activity equivalent of 0.1 mg. of hydrocortisone (one unit) per ml. as determined by the glycogen deposition test (Pabst, Sheppard and Kuizenga, 1947). A volume of 20 ml. per 24 hours was injected into the jugular vein of each rat by means of a continuous injection machine which was incorporated into the fatigue apparatus. By means of this apparatus 12 rats can be studied simultaneously.

EXPERIMENTS AND RESULTS

In each of the following experiments comparisons of replacement therapy were made simultaneously.

Each average value shown in Figure 1 for experiments 1, 2, and 3 is based upon 16 rats.

Experiment 1 involved hypophysectomized rats. One rat of each pair

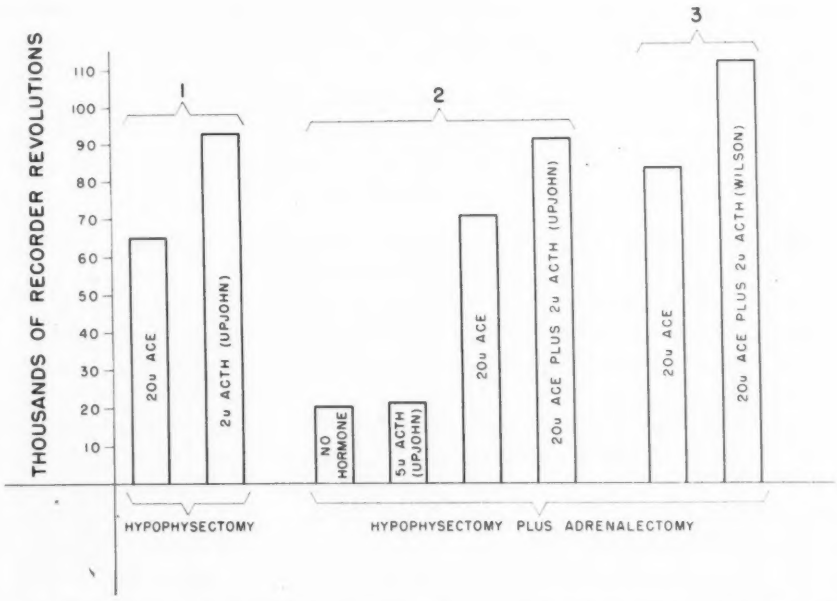

FIG. 1. The effect of some preparations of hormones in the hypophysectomized rat in the presence and absence of the adrenal glands. Averages. See METHODS for definition of "units."

was given 20 units of adrenal cortical extract per 24 hours per rat. The second rat of each pair was given 2 units of corticotropin (Upjohn) per 24 hours per rat. The average total work performed by the rats given corticotropin was significantly greater than the total work performed by the rats given adrenal cortical extract.

Experiment 2 involved 4 groups of hypophysectomized-adrenalectomized rats. The untreated rats worked very poorly; rats given 5 units of corticotropin (Upjohn) per 24 hours failed to work significantly better than the untreated controls; rats given 20 units of adrenal cortical extract per 24 hours showed improved but subnormal performance; and rats given 20 units of adrenal cortical extract plus 2 units of corticotropin (Upjohn) per 24 hours showed a significantly greater average amount of work which was within the range of normal performance.

Experiment 3 involved 2 groups of hypophysectomized-adrenalectomized rats. Rats given 20 units of adrenal cortical extract per 24 hours showed improved but subnormal performance and rats given 20 units of adrenal cortical extract plus 2 units of corticotropin (Wilson) per 24 hours showed a significantly greater average amount of work which was within the range of normal performance.

Table 1 summarizes the results of experiments 4, 5, 6, and 7 together with the number of rats per group, the averages and variability of the averages.

In experiment 4, sham hypophysectomized rats performed normal amounts of work; untreated hypophysectomized rats worked very poorly; and hypophysectomized rats treated with 2 units of corticotropin (Upjohn) per 24 hours performed normal amounts of work.

TABLE 1. EXTRA-ADRENAL EFFECT OF SOME PREPARATIONS OF CORTICOTROPIN UPON MUSCLE WORK IN THE RAT

Experiment	Experimental condition	Number rats	Treatment		Total average recorder revolutions
			Hormone	Dose/24 hrs.	
				Units[1]	
4	sham hypophysectomy	15	saline only	0	91,980±3,002
	hypophysectomy only	15	saline only	0	21,548±1,443
	hypophysectomy only	12	corticotropin (Upjohn)	2	93,013±3,529
5	hypophysectomy only	15	adrenal cortical extract	5	58,594±3,631
	hypophysectomy only	15	hydrocortisone	5	70,711±5,145
	hypophysectomy only	16	adrenal cortical extract	10	62,998±4,809
	hypophysectomy only	16	hydrocortisone	10	75,220±3,948
	hypophysectomy only	16	adrenal cortical extract	20	68,586±5,243
	hypophysectomy only	16	hydrocortisone	20	75,690±5,043
	hypophysectomy only	12	adrenal cortical extract	40	65,742±4,829
6	hypophysectomy & adrenalectomy	16	adrenal cortical extract	20	72,400±2,735
	hypophysectomy & adrenalectomy	16	adrenal cortical extract plus corticotropin—(Armour)	20 5	107,094±3,931
7	adrenalectomy only	16	adrenal cortical extract	20	102,172±3,275
	adrenalectomy only	17	adrenal cortical extract plus corticotropin—(Upjohn)	20 2	100,941±2,549

[1] The dosage of corticotropin is expressed as U.S.P. units. The dosage of adrenal cortical extract and of hydrocortisone is expressed as glycogen deposition units in which 1 unit is the activity equivalent of 0.1 mg. of hydrocortisone.

Experiment 5 involved hypophysectomized rats. Comparisons were made of the efficacy of hydrocortisone and adrenal cortical extract given at dosage levels of 5, 10, and 20 units per 24 hours per rat. The work output was enhanced above that of untreated rats and showed a positive relationship to dose but the performance remained subnormal. The response to hydrocortisone was greater than the response to adrenal cortical extract at each dosage level. When the dose of adrenal cortical extract was increased to 40 units per 24 hours the work output was not increased.

Experiment 6 involved hypophysectomized-adrenalectomized rats. Rats given 20 units of adrenal cortical extract per 24 hours showed improved but subnormal performance whereas rats given 20 units of adrenal cortical extract plus 5 units of corticotropin (Armour) per 24 hours showed normal performance.

Experiment 7 involved adrenalectomized rats. The administration of 20 units of adrenal cortical extract per 24 hours supported normal performance which was not surpassed by the administration of 20 units of adrenal cortical extract plus 2 units of corticotropin (Upjohn) per 24 hours in a second group of rats.

DISCUSSION

Adrenal cortical extract will fully correct the incapacity to work which develops following adrenalectomy in the rat (Ingle and Nezamis, 1949; experiment 7, Table 1 this paper) but it does not fully sustain the ability of the hypophysectomized rat to work (experiment 1, Figure 1; experiment 5, Table 1). These facts indicate that the anterior pituitary secretes some principle essential for normal vigor which does not act solely via the adrenal cortices.

Such a principle is present in each of the three commercial preparations of corticotropin which we have studied. These fractions of pituitary extracts will fully correct the incapacity to work which develops following hypophysectomy and when added to optimal amounts of adrenal cortical extracts will restore work output of the hypophysectomized-adrenalectomized rat to normal.

The possible explanations for the extra-adrenal effects of these samples of corticotropin are as follows. 1. The extra-adrenal action may be due to contamination of the corticotropin by a known hormone of the anterior pituitary gland. 2. The extra-adrenal action may be due to contamination of corticotropin by some unknown principle. 3. The biologic effects of pure corticotropin may not be limited to the adrenal cortices.

The administration of corticotropin alone did not significantly improve the work performance of the hypophysectomized-adrenalectomized rat

(experiment 2, Fig. 1). The extra-adrenal effect was evident only in animals which received adrenal cortical extract and were no longer adrenally insufficient. This can be added to a growing list of biologic responses for which the adrenal cortical hormones play a "permissive" role (Ingle, 1952.)

In experiment 5 (Table 1), hydrocortisone was found to be superior to adrenal cortical extract in supporting work performance of hypophysectomized rats when doses were matched on the basis of glycogen deposition units. In the adrenalectomized rat adrenal cortical extract is superior to hydrocortisone in supporting the ability of the rat to work (Ingle, Nezamis and Morley, 1952). It is possible that the basal secretion of hormones by the hypoactive adrenal cortices of the hypophysectomized rat is able to compensate for such qualitative deficiency of hydrocortisone as is observed in adrenalectomized rats.

Inspection of the average values for work by animals in separate experiments shows some significant variations in results when procedures and treatment are uniform but time is a variable. This emphasizes the necessity for the simultaneous comparison of the control and experimental groups within each individual experiment. The most important incompletely controlled variable in our experiments which relates to time is that of the room temperature to which the rats are exposed during surgery. Although air conditioned, the room may vary from 22 to 27° C. from one period of time to another. Animals which are exposed at the lower range of temperature during surgery tend to perform less work than do animals maintained at optimal temperature.

SUMMARY

Male rats of 200 gms. weight were anesthetized with barbiturates and following operation were subjected to faradic stimulation of the gastrocnemius muscle to lift a 100 gm. weight 5 times per second. Stimulation was continued until the death of the animal or for 48 hours. Hormones were administered by continuous intravenous injection. The following points are established. 1. The subnormal performance of adrenalectomized rats can be restored to normal by adrenal cortical extract. 2. The subnormal performance of hypophysectomized rats is restored to normal by certain preparations of corticotropin. 3. The performance of hypophysectomized rats and of hypophysectomized-adrenalectomized rats is improved but is not restored to normal by adrenal cortical hormones. 4. The performance of hypophysectomized rats and of hypophysectomized-adrenalectomized rats is restored to normal by adrenal cortical extract plus certain preparations of corticotropin. 5. Corticotropin does not improve the performance of hypophysectomized-adrenalectomized rats in the absence of adrenal

cortical hormones and it does not improve the performance of adrenalectomized rats which have the pituitary gland intact. These experiments indicate that the pituitary gland secretes a principle having an extra-adrenal action upon body economy which is present in certain preparations of corticotropin and may or may not be identical with the principle(s) which affect body economy via the adrenal cortex itself.

Acknowledgment

We wish to express our thanks to Mr. J. W. Nelson, Department of Endocrinology, The Upjohn Company, who supplied one preparation of corticotropin (Upjohn) and to Dr. E. D. Nielson, Department of Endocrinology, the Upjohn Company, who supplied the hydrocortisone.

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RELATIONSHIP OF THE ADRENAL GLANDS TO THE DIABETOGENIC EFFECT OF ETHYLENEDIAMINE IN THE PARTIALLY DEPANCREATIZED RAT

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THE injection of ethylenediamine has been found to cause exacerabtion of the diabetes of the partially depancreatized force-fed rat during the period of its injection (Ingle, Beary and Purmalis, 1952).

The data of the present report show that ethylenediamine has an extra-adrenal effect upon glycosuria but that the response to this noxious agent is modified by removal of the adrenal glands.

METHODS

Male rats of the Sprague-Dawley strain were obtained from the Upjohn breeding colony and were maintained on Archer Dog Pellets until they were partially depancreatized at a weight of 275 to 280 gm. After diabetes was established all of the rats were placed in metabolism cages and force-fed a medium carbohydrate diet (Ingle, 1952a) by stomach tube each morning (8:30 to 9:30 A.M.) and afternoon (4:15 to 5:00 P.M.). The techniques and diet were modifications of those described by Reinecke, Ball and Samuels (1939). Asepsis was preserved during the surgical procedures (Ingle and Griffith, 1942). The adrenal cortical extract (ACE) was prepared from mixed hog and beef adrenal glands and contained the biological activity equivalent to 0.1 mg. of hydrocortisone (1 glycogen unit) per ml. as assayed by the liver glycogen deposition test (Pabst, Sheppard and Kuizenga, 1947). It was administered by subcutaneous injection twice daily. Ethylenediamine, a liquid, was administered by subcutaneous injection.

The animals were kept at a temperature of 74 to 78° F. Twenty-four-hour samples of urine were collected at the same hour (8:00 to 8:30 A.M.) and were preserved with toluene. Urine glucose was determined by the method of Shaffer and Williams (1935) and blood glucose by the method of Miller and Van Slyke (1936).

EXPERIMENTS AND RESULTS

The effect of ethylenediamine upon the level of blood glucose in the partially depancreatized rat is shown in experiment 1 (Fig. 1). Six mildly diabetic rats were given 0.05 ml. of ethylenediamine once daily for 5 days and twice daily for 5 days. Samples of tail blood were taken just prior to the morning feeding and 2, 4 and 6 hours following the morning feeding during the control period and again on the 10th day that ethylenediamine was injected. The level of blood glucose in each rat was elevated above

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control values at each period during the injection of ethylenediamine. On the day that the control blood samples were taken the 6 animals excreted an average of 1398 mg. of glucose and on the day that the experimental blood samples were taken the rats excreted an average of 3,405 mg. of glucose. These results show that the increase in urinary glucose is accompanied by further elevation of the blood glucose and that the increased excretion of glucose during the administration of ethylenediamine does not represent renal glycosuria.

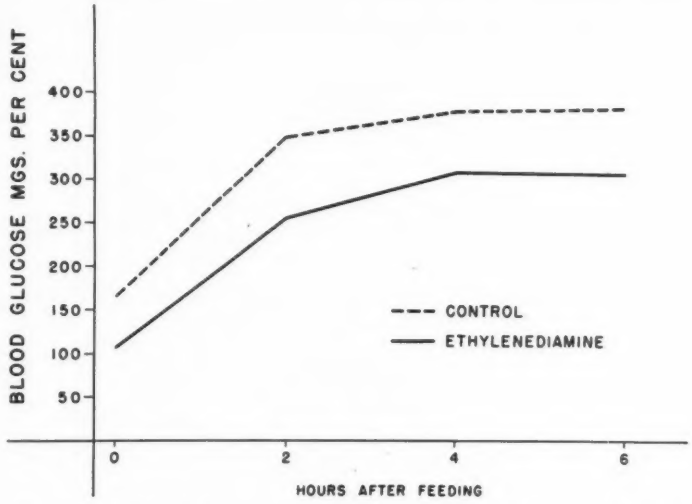

FIG. 1. The blood glucose of 6 mildly diabetic rats before and during administration of ethylenediamine. Averages.

In experiment 2 (Fig. 2), 7 moderately diabetic rats were subjected to adrenal enucleation (demedullation). A minimum period of 60 days was allowed for regeneration of the adrenal cortices. The animals were then given 0.05 ml. of ethylenediamine once daily for 5 days and twice daily for 5 days. The level of urinary glucose was increased significantly during the injection of ethylenediamine in each rat. When the injections were stopped the glycosuria fell to the preinjunction level. This experiment shows that ethylenediamine affects the level of glycosuria in the absence of the adrenal medullae.

Experiment 3 (Fig. 3) involved 13 moderately diabetic rats. Seven rats were adrenalectomized and treated with 3 ml. daily of ACE. Six rats were subjected to sham-adrenalectomy and given control injections of 3 ml. of 0.9 per cent sodium chloride solution. All of the animals were given 0.05

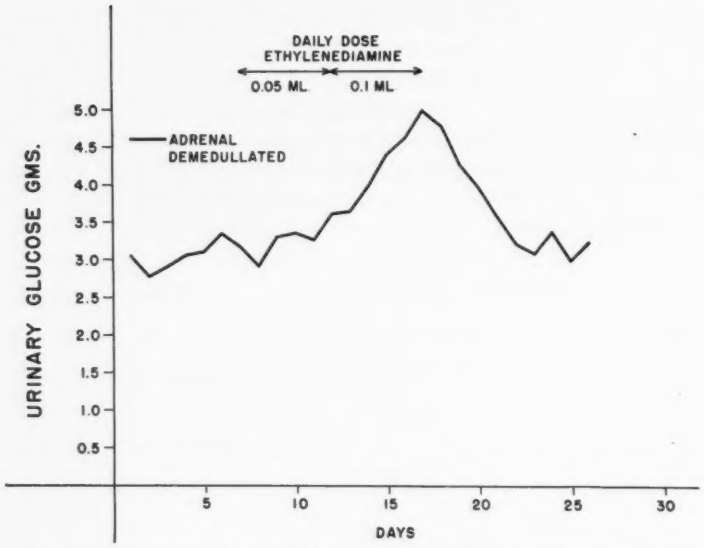

FIG. 2. Diabetogenic effect of ethylenediamine in 7 adrenal demedullated, partially depancreatized rats. Averages.

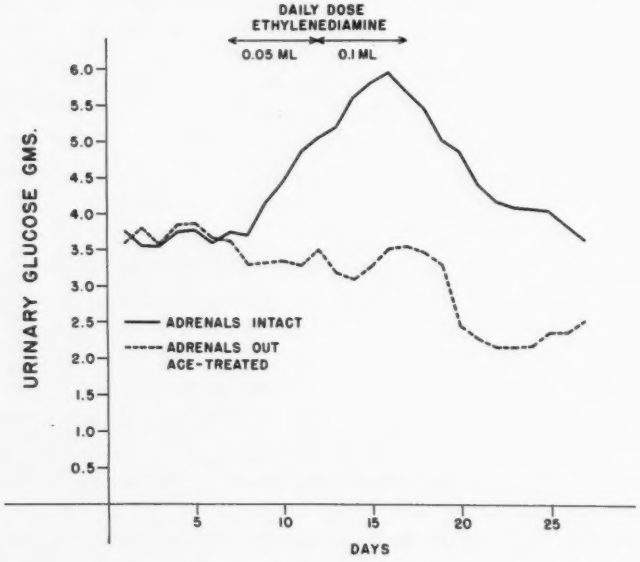

FIG. 3. Effects of ethylenediamine upon the glycosuria of partially depancreatized rats in the presence and absence of the adrenal glands. Seven adrenalectomized rats treated with 3 ml. ACE daily. Six non-adrenalectomized rats. Averages.

ml. of ethylenediamine once daily for 5 days and twice daily for 5 days. There was a striking increase in the glycosuria of the non-adrenalectomized rats but there was no increase in the glycosuria of the adrenalectomized rats. When the injection of ethylenediamine was stopped, the glycosuria of the non-adrenalectomized rats fell to the pre-injection level whereas the glycosuria of the adrenalectomized rats fell below the pre-injection level.

At this point in the study it seemed probable that the diabetogenic activity of ethylenediamine was mediated by an increase in the secretory activity of the adrenal cortices since this noxious agent failed to exacerbate the diabetes of adrenalectomized rats on a fixed intake of ACE. However,

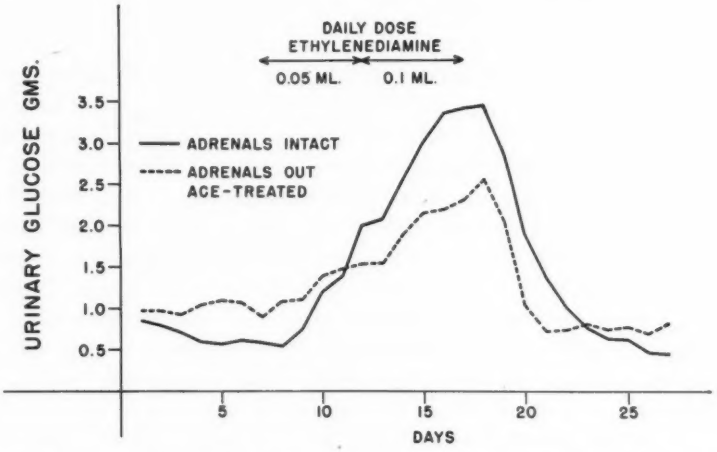

FIG. 4. Extra-adrenal effect of ethylenediamine. Six adrenalectomized rats treated with 5 ml. ACE daily. Six non-adrenalectomized rats. Averages.

this is not the only interpretation that can be made of the results. It is known that diabetes is ameliorated by adrenal cortical insufficiency and hence the action of diabetogenic agents is masked. It is also known that adrenalectomized animals maintained on a fixed intake of ACE just adequate to maintain eucorticalism under non-stress conditions can become adrenally insufficient during exposure to severe stressors. On the basis of these considerations it was postulated that ethylenediamine may have an extra-adrenal diabetogenic effect which is masked by the concomitant production of a state of adrenal cortical insufficiency caused by the toxicity of the substance. According to this point of view the secretory response of the adrenal cortex to a stressor may serve as the supporting cause of the metabolic response without becoming the inciting cause.

Experiment 4 (Fig. 4) served as a partial test of the foregoing hypothesis.

Very mildly diabetic rats were used for the reason that they are more sensitive to diabetogenic agents than are animals having more severe diabetes. Six rats were subjected to sham-adrenalectomy and given control injections of saline. Six additional rats were adrenalectomized and treated with 5 ml. of ACE daily. This amount is greater than the dosage used in experiment 3. All of the rats were given 0.05 ml. of ethylenediamine once daily for 5 days and twice daily for 5 days. There was exacerbation of the diabetes in all of the rats of each group but the average response was greater in the non-adrenalectomized rats. This experiment shows that ethylenediamine does have an extra-adrenal effect upon the level of glycosuria under the conditions of this experiment.

DISCUSSION

The data of these experiments have some bearing upon current concepts of the role of the adrenal cortex in homeostasis. According to one hypothesis (Selye, 1952) the increased secretory activity of the adrenal cortices during stress or an imbalance in the secretion of the adrenal cortical hormones during stress can cause metabolic abnormalities and disease. According to the second concept (Ingle, 1952b; Ingle and Baker, 1953) the increased secretory activity of the adrenal cortices during stress serves to meet an increased need for the hormones and does not represent hypercorticalism; the adrenal cortical hormones play a supporting or permissive role in certain metabolic and morphologic adjustments to stressors and in the manifestation of certain diseases but only rarely does the adrenal cortex play a primary role in the etiology of disease.

The words "permissive" and "supporting" have been used to characterize the relationship of the cortical hormones to certain metabolic responses which fail to become overt in adrenally insufficient animals but which reappear when the adrenalectomized animal is treated with a fixed intake of ACE in doses adequate to maintain a state of eucorticalism. In this situation the response cannot have been caused by an increase in the secretory activity of the adrenal cortices for they were absent. It is concluded that the inciting cause of the response is extra-adrenal but that a state of eucorticalism is necessary to support biologic responsiveness. The data of this paper require a more complex explanation but it seems possible to hold to the same basic concept that a state of eucorticalism is necessary to support normal and even some pathologic responses to stimuli which are primarily extra-adrenal in action.

The fact that the cortical hormones play a permissive or supporting role in certain metabolic responses which fail to occur in the adrenally insufficient animal was first recognized by Ingle (1942) although the data of several earlier reports fit this concept (for review see Ingle, 1952b).

Actually this general concept can be extended equally well to other hormones and to other biologic principles such as vitamins. Admittedly, we need more meaningful and more precisely defined terms than "permissive" and "supporting."

It has become increasingly apparent that the supporting role of the cortical hormones is not an all-or-none effect. When the adrenalectomized animal is given a fixed intake of ACE sufficient to maintain a state of eucorticalism under non-stress conditions the response to mild stressors such as fractures may be full-blown (Ingle, Ward and Kuizenga, 1947). When similar animals are exposed to severe stressors it can be anticipated that hypocorticalism will ensue and that metabolic responses requiring a state of eucorticalism for support will be suppressed. This concept was expressed 10 years ago (Ingle, 1943) but it is only recently that we have obtained supporting data (Ingle and Nezamis, 1950; Figs. 3 and 4, this paper).

The fact that the cortical hormones play a permissive or supporting role in a few responses to extra-adrenal causes is established. The hypothesis that these hormones play a supporting rather than a regulatory role in most metabolic adjustments seems tenable but is not proven. The facts provide some basis for the hypothesis that the cortical hormones play a supporting rather than an etiologic role in certain diseases such as hypertension and diabetes (Ingle, 1952b; Ingle and Baker, 1953) but at the present time our considerations of cause and effect relationships should only serve to stimulate further experimentation.

We need to think critically about the meaning of the word "cause." According to our hypothesis, the secretory response of the adrenal cortex to a stressor serves to sustain a state of eucorticalism and thereby supports the metabolic responsiveness. The response of the adrenal cortex may be a necessary antecedent event for certain metabolic adjustments to severe stressors but it is a supporting and not an inciting cause. The number of necessary antecedent events (causes) of a biologic response may be great and not subject to complete analysis. Together they form a responsive pattern or gestalt upon which inciting causes can act. Our thinking about such problems is limited by lack of knowledge and a limited comprehension span and also by habits of over-simplification.

SUMMARY

These studies were designed to test the hypothesis that ethylenediamine causes exacerbation of diabetes in the partially depancreatized rat via activation of the anterior pituitary-adrenal cortex axis. Partially depancreatized male rats were force-fed a medium carbohydrate diet by stomach tube. Ethylenediamine was injected in amounts of 0.05 ml. once

daily for 5 days and twice daily for 5 days in each of 4 experiments. In experiment 1, ethylenediamine was found to cause a rise in the level of blood glucose as well as in urinary glucose. In experiment 2, ethylenediamine caused exacerbation of the diabetes of adrenal demedullated rats. In experiment 3, moderately diabetic adrenalectomized rats treated with 3 glycogen units daily of ACE failed to show exacerbation of the diabetes during the injection of ethylenediamine. It was postulated that ethylenediamine (a noxious agent) may have an extra-adrenal diabetogenic action which is masked by the development of adrenal cortical insufficiency in rats on a fixed intake of ACE adequate to meet the needs of non-stressful conditions only. Experiment 4 was a partial test of this hypothesis. Very mildly diabetic adrenalectomized rats were treated with 5 glycogen units of ACE per day. The injection of ethylenediamine caused exacerbation of the diabetes in each of the adrenalectomized rats but the response was greater in nonadrenalectomized animals. These experiments show that ethylenediamine has an extra-adrenal effect upon glycosuria but that the response is modified by removal of the adrenal glands. It is suggested that the secretory response of the adrenal cortices during the injection of ethylenediamine tends to maintain a state of eucorticalism which is essential for biologic responsiveness.

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ALTERATIONS IN ORGAN GLYCOGEN RESERVES AND RESPIRATION FOLLOWING EPINEPHRINE[1]

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IT IS an established fact that epinephrine mobilizes the carbohydrate stores of the body (Cori, 1931 and 1940; Britton and Silvette, 1937). Nevertheless the marked alterations in carbohydrate metabolism in the adrenalectomized animal, usually demonstrated in liver and muscle, have been attributed generally to the absence of the cortical hormones. It has been shown by Strand and Gordon (1950) that carbohydrate levels within the lymphoid organs of the rat also are influenced by the secretions of the adrenal gland, with either cortical hormone or epinephrine, administered daily from 1–3 weeks, capable of restoring the depleted glycogen stores resulting from adrenalectomy. Cori and Cori (1928) have demonstrated that a single injection of epinephrine, administered to fasted rats, results in an increase in liver glycogen within a 4-hour period. The present experiments attempt to determine the acute and chronic effects of epinephrine on thymus, spleen, liver, muscle and blood in the fasted adrenalectomized and sham-adrenalectomized animal; the influence of hypophysectomy upon such epinephrine action also will be described. Respiration studies accompany glycogen determinations for each tissue, except blood, while glycogen is localized histochemically in the livers and lymph nodes of all groups under observation.

MATERIALS AND METHODS

Male rats of a hardy, inbred strain, weighing from 120–170 gm. at the time of operation were used. The animals were maintained on the standard laboratory rations, with the addition of evaporated milk to the diet of the hypophysectomized rats and 1% saline as drinking water for the adrenalectomized animals. All animals were kept at temperatures of 70–80 degrees F. The three main groups of rats established were adrenalectomized, sham-adrenalectomized and hypophysectomized; these were used in the acute and chronic experiments as follows:

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[1] Data taken from a dissertation in the Department of Biology submitted by Fleur L. Strand in partial fulfillment of the requirements for the degree of Doctor of Philosophy at New York University.

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A. *Acute Experiments*

Series I. *Sham-adrenalectomized and control animals.* This group consisted of 30 sham-adrenalectomized rats given 1 cc. of a 1:5000 solution of epinephrine (200 μg. adrenalin hydrochloride, Parke, Davis and Co.) 2 weeks after the operation. These animals were killed in groups of 6 each, at 1, 4, 13, 24 and 48 hours, respectively, after the single injection of epinephrine. Six control sham-adrenalectomized rats received no epinephrine.

Series II. *Adrenalectomized and control animals.* A total of 30 adrenalectomized rats was killed in groups of 6 each, at 1, 4, 13, 24 and 48 hours after the administration of 200 μg. epinephrine, 2 weeks subsequent to the operation. Six control adrenalectomized animals did not receive epinephrine.

Series III. *Hypophysectomized and control animals.* A total of 30 hypophysectomized rats, operated on 2 to 3 weeks previously, was killed at 1, 4, 13, 24 and 48 hours after the administration of 200 μg. epinephrine, in groups of 6 for each time interval. Six control hypophysectomized animals received no epinephrine.

B. *Chronic Experiments*

In this series, 6 sham-adrenalectomized, 6 adrenalectomized and 6 hypophysectomized rats were injected daily for 2 weeks with 200 μg. epinephrine. In the case of the hypophysectomized rats, it was found necessary to reduce the dosage to 100 μg. for the first 3 days of treatment. It was then possible to revert to the full dose without mortalities.

C. *Dosage Effects*

Adrenalectomized rats were killed 1 hour after the administration of 1) 100 μg. epinephrine, 2) 50 μg. epinephrine, 3) 40 μg. epinephrine. Twelve animals were used for this study.

Adrenalectomy and hypophysectomy were performed under light ether anesthesia and the operated animals were checked for completeness of the surgery at autopsy. All rats were fasted from 20 to 24 hours before sacrifice, drinking water being provided *ad lib.* The techniques of Good, Kramer and Somogyi (1933) and Shaffer and Somogyi (1933) were employed for glycogen determinations in the mesenteric lymph nodes, thymus, spleen, liver, gastrocnemius muscle and cardiac blood of all experimental animals. Determinations of respiratory activity were executed on these same organs, except blood, using the reduction of 2,3,5 triphenyl tetrazolium chloride (Black and Kleiner, 1949) as the criterion.

Glycogen was localized histochemically in the iliac lymph nodes and liver, these tissues being fixed in Rossman's fluid according to Bensley (1938). The staining technique was that recommended by McManus (1946), using periodic acid and the Schiff reagent, followed by rinsings in sulphurous acid. With this method the glycogen granules stain a purplish red. Control sections, incubated for 30 minutes in saliva, were run for all sections.

RESULTS[3]

A. *Acute Experiments*

All effects described are depicted in Tables 1 and 2.

[3] All statements made in this section regarding differences among the means of the different groups of animals compared are based on statistical analyses for probability values, derived from the distribution of Fisher's t. P values of <0.05 have been considered significant.

TABLE 1. LYMPHOID GLYCOGEN (MEAN% ± STANDARD ERRORS) AND RESPIRATION[1] (INTENSITY OF COLOR/MG. DRY WT. ± STANDARD ERRORS)

Hours after epinephrine (200 μg.)	Sham adrenalectomy			Adrenalectomy			Hypophysectomy		
	Lymph node	Thymus	Spleen	Lymph node	Thymus	Spleen	Lymph node	Thymus	Spleen
0[2]	0.451±0.022 (50.2±2.2)	0.353±0.041 (22.0±1.4)	0.209±0.021 (33.9±1.4)	0.165±0.018 (61.4±3.8)	0.143±0.027 (38.4±0.4)	0.120±0.012 (42.7±1.4)	0.078±0.013 (57.0±2.4)	0.197±0.020 (27.9±2.0)	0.235±0.015 (44.1±2.2)
1	0.280±0.088 (15.6±2.2)	0.231±0.013 (18.9±2.0)	0.088±0.006 (16.5±1.9)	0.049±0.010 (32.7±2.5)	0.040±0.016 (19.7±2.0)	0.050±0.014 (36.3±1.7)	0.025±0.006 (26.2±1.6)	0.133±0.005 (17.7±0.8)	0.072±0.011 (28.4±1.0)
4	0.179±0.022 (29.5±4.6)	0.102±0.016 (21.0±3.0)	0.397±0.032 (19.0±1.5)	0.135±0.017 (18.2±1.7)	0.133±0.016 (14.6±1.1)	0.102±0.011 (18.8±1.0)	0.104±0.008 (43.1±3.1)	0.125±0.009 (33.0±2.0)	0.081±0.018 (32.6±2.0)
13	0.324±0.053 (42.4±1.7)	0.128±0.013 (17.7±0.9)	0.267±0.013 (19.8±1.1)	0.177±0.016 (17.4±1.8)	0.228±0.034 (14.3±1.2)	0.178±0.020 (15.2±1.0)	0.041±0.007 (42.6±1.0)	0.103±0.005 (26.6±2.6)	0.040±0.005 (33.3±0.7)
24	0.254±0.082 (59.3±7.0)	0.187±0.018 (33.9±2.5)	0.128±0.013 (35.5±2.8)	0.182±0.014 (50.0±2.4)	0.140±0.018 (30.6±1.4)	0.124±0.008 (40.8±1.3)	0.521±0.038 (40.7±1.1)	0.474±0.038 (25.2±1.3)	0.315±0.018 (20.7±1.2)
48	0.430±0.022 (50.8±2.7)	0.421±0.031 (20.8±1.2)	0.202±0.022 (32.5±0.9)	0.134±0.011 (53.0±1.6)	0.110±0.014 (28.5±1.0)	0.221±0.010 (33.9±1.4)	0.079±0.012 (53.6±1.8)	0.191±0.020 (28.4±1.2)	0.244±0.015 (41.2±1.8)
2 weeks[3]	0.642±0.063 (45.8±3.0)	0.340±0.042 (27.0±1.7)	0.221±0.031 (27.5±2.3)	0.147±0.003 (45.2±2.3)	0.150±0.015 (26.8±1.9)	0.142±0.014 (35.9±2.8)	0.270±0.032 (40.7±1.3)	0.450±0.034 (32.8±1.6)	0.225±0.032 (64.6±4.1)

[1] Indicated in parentheses below each glycogen value.
[2] No epinephrine administered.
[3] Epinephrine given daily for 2 weeks.

TABLE 2. LIVER, MUSCLE AND BLOOD GLYCOGEN[4] (MEAN% ± STANDARD ERRORS) AND RESPIRATION[1] (INTENSITY OF COLOR/MG. DRY WT. ± STANDARD ERRORS)

Hours after epinephrine (200 μg.)	Sham adrenalectomy			Adrenalectomy			Hypophysectomy		
	Liver	Muscle	Blood	Liver	Muscle	Blood	Liver	Muscle	Blood
0[2]	1.260±0.170 (69.6±3.5)	0.200±0.028 (32.7±1.0)	0.088±0.004	0.532±0.042 (86.3±1.1)	0.124±0.009 (37.6±1.6)	0.023±0.001	0.024±0.008 (71.2±1.5)	0.161±0.014 (14.6±1.5)	0.075±0.005
1	0.219±0.032 (39.1±2.4)	0.124±0.014 (25.4±1.0)	0.020±0.004	0.352±0.037 (76.4±1.8)	0.076±0.019 (23.5±1.6)	0.025±0.002	0.093±0.011 (48.3±1.6)	0.072±0.011 (11.3±0.8)	0.017±0.002
4	2.144±0.057 (36.4±3.3)	0.126±0.008 (28.4±1.2)	0.075±0.003	1.237±0.080 (22.7±0.9)	0.085±0.004 (18.4±1.1)	0.051±0.004	0.141±0.026 (71.5±1.9)	0.081±0.010 (22.6±2.1)	0.014±0.001
13	1.283±0.408 (54.0±2.8)	0.097±0.010 (29.2±2.4)	0.024±0.001	1.145±0.144 (23.9±2.1)	0.177±0.015 (13.1±1.3)	0.018±0.002	0.055±0.007 (75.4±3.1)	0.076±0.005 (16.6±1.0)	0.019±0.001
24	0.045±0.005 (69.7±5.3)	0.217±0.029 (26.5±4.3)	0.048±0.004	0.257±0.031 (87.2±4.4)	0.166±0.011 (28.0±1.4)	0.029±0.001	0.190±0.032 (67.6±1.7)	0.228±0.033 (19.1±1.2)	0.061±0.003
48	1.042±0.030 (69.4±2.6)	0.247±0.015 (36.7±1.3)	0.088±0.003	0.457±0.079 (86.1±2.4)	0.263±0.015 (23.1±1.2)	0.026±0.002	0.035±0.006 (72.3±3.1)	0.166±0.018 (16.3±1.1)	0.079±0.005
2 weeks[3]	0.249±0.032 (90.0±4.9)	0.336±0.025 (25.1±1.3)	0.027±0.003	0.745±0.039 (71.5±3.3)	0.165±0.022 (28.5±3.2)	0.027±0.001	0.196±0.021 (92.3±2.6)	0.288±0.024 (15.3±1.7)	0.053±0.004

[1] Indicated in parentheses below each glycogen value.
[2] No epinephrine administered.
[3] Epinephrine given daily for 2 weeks.
[4] Specific gravity of blood calculated as 1.027.

1. *Organ Glycogen and Respiration Prior to Epinephrine Administration*

Series I. *Sham-adrenalectomized animals.* The liver glycogen concentration of the fasted, sham-adrenalectomized rat is approximately 3 to 6 times as great as that of lymphoid organs and muscle. There are only slight differences in the glycogen levels within the various lymphoid organs tested, with lymph node glycogen being the greatest. The concentration of blood glycogen is about one-fifteenth that of liver. Thus in the fasted, sham-adrenalectomized rat, organs placed in order of increasing glycogen content are blood, muscle-spleen, thymus, lymph node and liver.

Series II. *Adrenalectomized animals.* Adrenalectomy results in a decrease in the glycogen levels within the lymph nodes, thymus, spleen, liver and muscle to approximately half those of the sham-operated rat. Blood glycogen is reduced to almost one quarter of its original level. Liver still contains the most, and blood the least glycogen, with the lymphoid tissues and muscle ranged in the same relative order of glycogen concentration as in the sham-operated animal.

Series III. *Hypophysectomized animals.* Hypophysectomy causes a more marked depletion in the glycogen reserves of the liver than does adrenalectomy. In the fasted, hypophysectomized rat, liver glycogen is reduced to about one-sixtieth of that in the liver of the fasted sham-operated rat and it is less than the glycogen concentration in any of the tissues tested. Hypophysectomy also is followed by a decrease in the glycogen content of lymph node, thymus and blood. Splenic and muscle glycogen values are not altered significantly by removal of the pituitary gland. The relative order of increasing glycogen concentration of the tissues of the hypophysectomized rat is liver, blood-lymph node, muscle, thymus and spleen. This is almost the reverse of the order found in the sham-operated animal.

The data indicate that the relative respiratory activity of tissue slices from sham-operated rats is, in increasing order, thymus, muscle-spleen, lymph node and liver. Adrenalectomy increases the respiratory activity of all these tissues. Hypophysectomy increases significantly the respiration of the lymphoid tissues while liver respiration is not affected. Muscle tissue slices show a marked decrease in respiratory activity following hypophysectomy.

2. *Changes in Lymphoid Glycogen and Respiration over an Observed Period of 1–48 Hours Following an Injection of 200 μg. Epinephrine*

Series I. *Sham-adrenalectomized animals.* The glycogen concentration in lymph node, thymus and spleen is decreased considerably 1 hour after a single injection of epinephrine. At four hours after the injection, glycogen concentration in the thymus has decreased still further but that in spleen

has increased to a level greater than that found in the untreated control. By 13 hours, the glycogen in the lymph nodes has been restored to almost normal levels and at 48 hours these levels are equivalent to those of the untreated, sham-adrenalectomized rat. Although the carbohydrate reserves within the thymus are replaced more slowly, these attain normal values by 48 hours.

One hour following the administration of epinephrine to fasted, sham-adrenalectomized rats, the respiratory activity of spleen and lymph node declines considerably and returns slowly to approximately normal levels by 24 hours. Thymus slices do not exhibit a significant decrease in respiratory activity at 1 hour after the injection, but respiration rises to levels slightly higher than the untreated controls by 24 hours. At 48 hours, the respiratory activity of thymus tissue slices has returned to normal.

Series II. *Adrenalectomized animals.* A significant decrease in lymphoid glycogen has occurred 1 hour after a single injection of epinephrine. By 4 hours after hormone administration the glycogen reserves of the 3 lymphoid organs tested are almost completely replenished and at 13 hours thymic and splenic glycogen has attained higher levels than those seen prior to epinephrine treatment. By 48 hours, however, this increase is no longer apparent and the carbohydrate content of the lymphoid organs is comparable to that of the untreated, adrenalectomized controls. Splenic glycogen values are somewhat elevated at the 48 hour period.

In the adrenalectomized rat, administered epinephrine, the respiratory activity of lymph node, thymus and spleen is decreased at 1 hour and continues to decrease until 13 hours after the injection. Despite a rise in respiratory activity evident at 24 and 48 hours, the values are still not as high as in the untreated, adrenalectomized control.

Series III. *Hypophysectomized animals.* In the hypophysectomized rat, administration of a single dose of epinephrine results in a decrease in the glycogen content of lymph node, thymus and spleen. Lymph node glycogen shows a tendency to rise at 4 hours following the injection, then is secondarily depleted at 13 hours, to be elevated considerably at 24 hours. At 48 hours, lymph node glycogen is restored to normal levels. After the initial depletion of glycogen from the thymus and spleen, the concentration rises at 24 hours to values higher than those of the untreated, hypophysectomized control. By 48 hours, glycogen within the thymus and spleen has descended to normal control levels.

In the hypophysectomized rat, the respiratory activity of lymph node, thymus and spleen declines 1 hour after epinephrine administration. In all the lymphoid organs an increase is seen over the subsequent observed periods, until at 48 hours respiration levels are approximately as high as in the untreated, hypophysectomized controls. The respiratory activity of

splenic tissues shows a secondary decrease at 24 hours after the injection, then is elevated to normal by 48 hours.

3. *Changes in Liver and Muscle Glycogen and Respiration and Blood Glycogen over an Observed Period of 1–48 Hours Following an Injection of 200 μg. Epinephrine*

Series I. *Sham-adrenalectomized animals.* There is a large decrease in the concentration of glycogen within the liver 1 hour after the administration of 200 μg. epinephrine, but by 4 hours glycogen values are considerably greater than in the untreated, sham-adrenalectomized animal. There is a secondary decrease in liver glycogen, most noticeable at 24 hours after the injection, but hepatic levels of this substance are normal by 48 hours. A slow depletion of the glycogen reserves of muscle occurs in the first 13 hours after the epinephrine administration, but by 24 hours the lost carbohydrate has been replaced. Blood glycogen alterations resemble, but to a lesser degree, those seen in liver. There is a primary depletion at 1 hour after hormone administration and a secondary loss at 13 hours. Blood glycogen levels are normal again at 48 hours.

Tissue slices of both liver and muscle, in the sham-adrenalectomized rat, demonstrate a decline in respiratory activity 1 hour after the injection of epinephrine, followed by a return to normal, control levels by 24-48 hours.

Series II. *Adrenalectomized animals.* In the adrenalectomized rat given a single dose of epinephrine, a decrease in liver glycogen concentration occurs at 1 hour, but by 4 hours after the injection the glycogen reserves of the liver are approximately twice as great as those of the untreated, adrenalectomized control. At 24 hours, liver is secondarily depleted, to return to levels characterizing the untreated, adrenalectomized rat by 48 hours. Muscle glycogen is similarly depleted, but is not replenished until 13 hours after epinephrine administration, at which time the glycogen levels are higher than in the untreated, adrenalectomized animal. Muscle glycogen levels are still high at 48 hours. Blood glycogen shows no decrease at 1 hour but is increased at 4 hours. There is a drop in blood glycogen at 13 hours, but by 48 hours the levels are approximately normal.

In the adrenalectomized rat, given epinephrine, liver and muscle slices reveal a decrease in respiratory activity 1 hour after the injection and attain still lower levels at 4 or 13 hours. At 24 and 48 hours, hepatic respiration has returned to levels seen in the untreated, adrenalectomized control, while muscle respiration is still low, despite some increase over the initial depression.

Series III. *Hypophysectomized animals.* In the hypophysectomized rat, liver glycogen increases for 4 hours after the administration of epinephrine. By 48 hours, liver glycogen has returned to the levels of the untreated, hypophysectomized animal. Glycogen levels in muscle remain low up to 24 hours after epinephrine, at which time the glycogen concentration becomes slightly higher than in the hypophysectomized controls. By 48 hours, muscle glycogen has returned to normal levels. Similarly, blood glycogen remains low in the hypophysectomized rat until 24 hours after epinephrine administration, at which time the glycogen approaches the concentration of the untreated, hypophysectomized controls.

Both liver and muscle slices, in the hypophysectomized rat, show a depression of respiratory activity 1 hour after a single dose of epinephrine, but values have returned to levels seen in the untreated, hypophysectomized control by 13 hours.

B. *Chronic Experiments*

Organ Glycogen and Respiration Following Administration of 200 μg. Epinephrine Daily for 2 Weeks

Effects described are shown in Tables 1 and 2.

Series I. *Sham-adrenalectomized animals.* The most significant change in the fasted, sham-adrenalectomized rat, following chronic administration of epinephrine, is the depletion of the glycogen reserves of liver and blood. Glycogen levels are elevated in lymph node and muscle, while glycogen concentrations in thymus and spleen are unchanged. Chronic administration of epinephrine to sham-adrenalectomized rats results in an increase in the respiratory activity of liver, and to a lesser extent, of thymus. The respiration of spleen and muscle is depressed, while that of lymph node is unchanged after 2 weeks of daily epinephrine injections.

Series II. *Adrenalectomized animals.* In the fasted, adrenalectomized rat, administered epinephrine daily for 2 weeks, liver glycogen is slightly increased. Glycogen levels in lymph node, thymus, spleen, muscle and blood are essentially unchanged from those noted in untreated, adrenalectomized controls. The respiratory activity of all the tissues tested i.e. lymph node, thymus, spleen, liver and muscle, is depressed after the chronic administration of epinephrine.

Series III. *Hypophysectomized animals.* Chronic administration of epinephrine to hypophysectomized rats increases the glycogen concentration in lymph node, thymus, liver and muscle, while glycogen levels in blood are slightly decreased. There is an elevation of the respiratory activity of thymus, spleen and liver, while lymph node respiration decreases. Muscle respiration remains unchanged.

C. *Lymph Node and Thymus Weights in Acute and Chronic Experiments*

Effects described are shown in Table 3.

Series I. *Sham-adrenalectomized animals.* Administration of a single dose of epinephrine to the sham-operated rat results in a decrease in the weight of the mesenteric lymph nodes, an effect apparent 4 hours after the injection. Between 13 and 24 hours following the hormone administration the weights of the mesenteric lymph nodes increase and by 48 hours they are approximately double those seen in the untreated, sham-adrenalectomized control. Chronic administration of epinephrine to the sham-operated rat results in atrophy of the mesenteric lymph nodes. Thymus weights are decreased at 4 and 13 hours following a single dose of 200 μg. epinephrine.

TABLE 3. MESENTERIC LYMPH NODE AND THYMUS WEIGHTS IN GM. (MEANS ± STANDARD ERRORS)

Hours after epinephrine (200 μg.)	Sham adrenalectomy		Adrenalectomy		Hypophysectomy	
	Lymph node	Thymus	Lymph node	Thymus	Lymph node	Thymus
0[1]	0.068±0.014	0.138±0.006	0.096±0.011	0.244±0.015	0.104±0.016	0.084±0.019
1	0.071±0.004	0.145±0.002	0.102±0.008	0.272±0.080	0.077±0.004	0.061±0.004
4	0.040±0.004	0.075±0.011	0.064±0.007	0.246±0.006	0.106±0.013	0.113±0.018
13	0.046±0.005	0.048±0.007	0.057±0.009	0.173±0.005	0.094±0.002	0.112±0.012
24	0.101±0.014	0.174±0.010	0.111±0.004	0.391±0.034	0.093±0.004	0.073±0.006
48	0.127±0.006	0.160±0.006	0.202±0.016	0.335±0.010	0.116±0.024	0.078±0.017
2 weeks[2]	0.033±0.005	0.106±0.016	0.116±0.010	0.230±0.024	0.065±0.006	0.061±0.007

[1] No epinephrine administered.
[2] Epinephrine given daily for 2 weeks.

By 24 hours, the weights have increased to levels higher than those of the untreated, sham-adrenalectomized rat and remain high up to 48 hours. Chronic administration of the medullary hormone causes a slight decrease in thymic weight.

Series II. *Adrenalectomized animals.* Adrenalectomy causes an insignificant increase in the weight of the mesenteric lymph nodes. Following a single injection of epinephrine, a decrease in lymph node weight is noticeable at 4 hours, in the adrenalectomized animal. By 24 hours, lymph node weights are increased to levels approximately normal and by 48 hours the nodes have hypertrophied to approximately twice the weight of those in the untreated controls. Chronic administration of epinephrine to adrenalectomized rats results in an insignificant increase in lymph node weight. An increase is noted in the weight of the thymus two weeks after adrenalectomy. A single dose of epinephrine administered to adrenalectomized rats causes a slight decrease in thymic weight at 13 hours, followed by a considerable increase over the weight of the untreated, adrenalectomized controls at 24 hours. Weights are still high at 48 hours. Chronic administration of epinephrine causes no change in thymic weight as compared to adrenalectomized controls.

Series III. *Hypophysectomized animals.* A slight, but statistically insigni-

ficant, increase is noted in the weight of the lymph nodes 2 to 3 weeks following hypophysectomy. No significant alterations in lymph node weight are noted at 1, 4, 13, 24 and 48 hours after a single dose of epinephrine. Chronic administration of epinephrine to the hypophysectomized rat results in a decrease in weight of the mesenteric lymph nodes. Two to three weeks after hypophysectomy a decrease in the weight of the thymus is evident. Administration of a single dose of epinephrine is attended by an insignificant decrease in thymic weight at 1 hour, followed by a slight increase from 4–13 hours. By 24 hours after epinephrine administration, thymic weights are comparable to those of the untreated, hypophysectomized controls. Chronic administration of epinephrine results in an insignificant decrease in thymic weight.

D. The Effects of Varied Dosages of Epinephrine on the Organ Glycogen and Respiration of Adrenalectomized Rats

Adrenalectomized rats, at 1 hour after 1 injection of 100 μg., 50 μg. or 40 μg. epinephrine responded with a depletion of the glycogen reserves of the tissues similar to that evoked by the standard 200 μg. dosage. The decrease in tissue respiration, at this time, is not seen subsequent to the injection of 50 μg. or 40 μg. epinephrine.

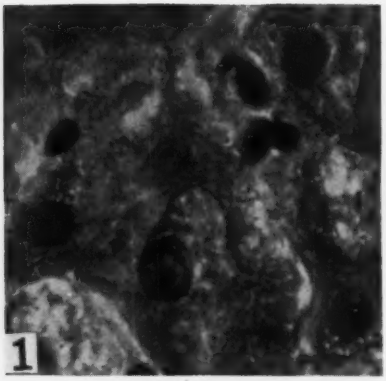
E. *Histochemical Localization of Glycogen in the Lymph Nodes and Liver*

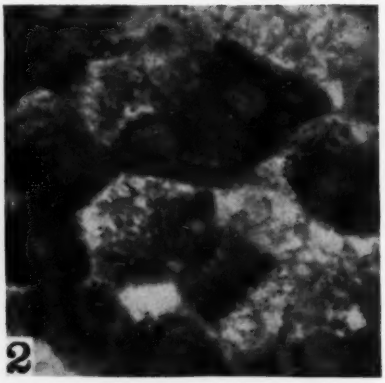
In the liver, the cytoplasm of the hepatic cells reveals purple staining granules in varying concentrations, roughly proportional to those determined chemically at each experimental period (Figs. 1 and 2). Glycogen is found localized in the cytoplasm of both the lymphocytes and plasma cells of the lymph nodes. As expected, the concentration of glycogen in these cells is much less than in the hepatic cells, but there is a definite purplish coloration of the cytoplasm of most lymphocytes and plasma cells, to varying degrees. This color does not appear in the saliva-treated controls. The macrophages also contain glycogen, which is visible in granular form but the density of the color is less than in the lymphocyte cytoplasm. The reticulum stains very lightly. The major changes demonstrated in the chemical experiments are confirmed by the histochemical studies (Figs. 3, 4 and 5).

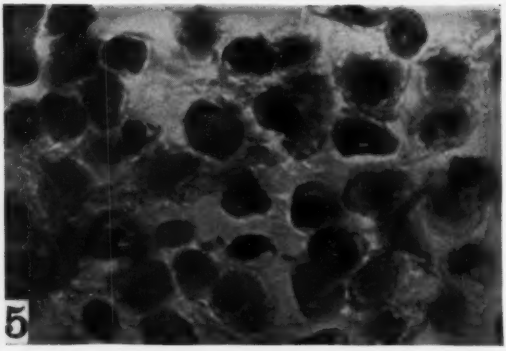
DISCUSSION

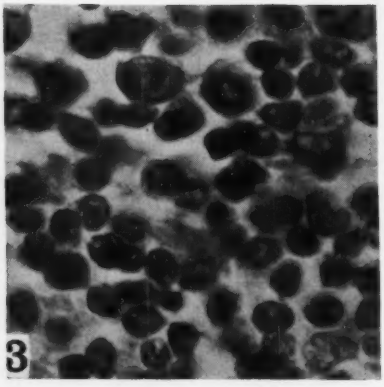
The present studies indicate that the lymphoid tissues constitute a site of glycogen reserve that may be mobilized for use within the organism. Due to the rapid turnover of lymphocytes within the lymphoid organs (Andreasen and Ottesen, 1945) the carbohydrate contribution of these organs attains a significance beyond their absolute weight.

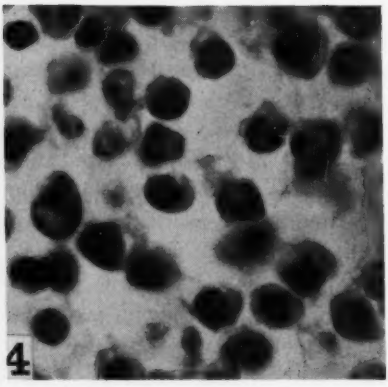
Although the initial action of the medullary hormone is to cause a de-











All figures represent photomicrographs of tissues fixed in Rossman's fluid, sectioned at 4 μ and treated with periodic acid and the Schiff reagent. Magnification 1300×.

FIG. 1. Liver of an adrenalectomized rat, showing a light scattering of glycogen granules in the cytoplasm of the hepatic cells.

pletion of the carbohydrate content of the tissues, this is followed by a replacement of these stores, which in most cases may be termed an "overcompensation" as glycogen values are elevated eventually beyond the untreated, control levels of each group. There are differences, however, in the extent and timing of the restorative processes in the sham-operated, adrenalectomized and hypophysectomized animals. The pattern of response of the hypophysectomized rats, despite differences in rate, follows that of the sham-operated animals too closely to support a theory that epinephrine action in this regard is accomplished through the pituitary gland in the intact animal. The hypothesis, advanced by Long and his collaborators (1945 and 1950), that epinephrine acts directly upon the anterior pituitary gland, cannot be extended to explain entirely the action of this agent on carbohydrate metabolism. The observation by Winternitz and Long (1952) that cortical hormone is required for significant replacement of liver glycogen stores in the adrenalectomized rat given epinephrine is not in accord with the present findings. These studies suggest a direct action of epinephrine on carbohydrate reserves, with the rate of this effect, rather than the quality of the action, altered by the concentration of cortical and hypophyseal hormones.

It may be seen from the tetrazolium studies on tissue slices that adrenalectomy or hypophysectomy induces an increase in the respiratory activity of all the tissues tested, except liver and muscle in the adrenalectomized rat. In addition, a depression of respiratory activity of the tissues of all experimental animals is detectable 1 hour after epinephrine administration, with a gradual return to normal levels.

The data indicate that the hypertrophy of the thymus following adrenalectomy is accompanied by a decrease in the glycogen concentration within it. Although hypophysectomy is usually considered to result in hypertrophy of the thymus (Ingle, 1938) pituitary ablation in these experiments led to a marked atrophy of the thymus, greater proportionally than the loss of weight in the other organs.

In all 3 groups of experimental animals the timing of the replacement of

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FIG. 2. Liver of an adrenalectomized rat, 13 hours after the administration of 200 μg. epinephrine, showing the marked increase in the concentration of the glycogen granules in the cytoplasm of the hepatic cells.

FIG. 3. Iliac lymph node of an adrenalectomized rat. Relatively little glycogen is visible in the cytoplasm of these cells.

FIG. 4. Iliac lymph node of an adrenalectomized rat, 24 hours after the administration of 200 μg. epinephrine. There is an increase in the number of lymphocytes and plasma cells containing glycogen.

FIG. 5. Iliac lymph node of a hypophysectomized rat, 24 hours after the administration of 200 μg. epinephrine. Glycogen is concentrated in the cytoplasm of most cells.

the glycogen reserves of the 6 tissues tested suggests a reciprocal relation between liver and blood glycogen and the lymphoid-muscle stores. The behavior of spleen is more like liver, probably due to its high peripheral blood content. Rough estimates of the amount of glycogen lost by lymphoid-muscle stores and gained by liver indicate that there are probably tissue depôts, in addition to those studied in this report, which are concerned with the storage and utilization of carbohydrate. Future studies should consider the relation of the glycogen reserves in adipose tissue (Engel and Scott, 1950) to those in other depôts of the body under the influence of hormone deficiencies and replacement therapy.

SUMMARY

1. The administration of a single dose of epinephrine (200 μg.) to sham-adrenalectomized, adrenalectomized and hypophysectomized fasted, male rats induces shifts in carbohydrate reserves and alterations in tissue respiratory activity over an observed period of from 1–48 hours. The general pattern of glycogen response in lymph nodes, thymus, spleen, liver and muscle is characterized by an initial depletion and replacement of these reserves followed, in some cases, by a secondary depletion and restoration. The different effects on glycogen reserves that occur in the 3 groups of tested rats may be explained better on the basis of differences in rate, rather than the type, of response.

2. Histochemical analysis of liver and lymph nodes reveals glycogen to be present within the cytoplasm of the hepatic cells and within most lymphocytes and plasma cells.

3. The decrease in glycogen reserves in lymphoid tissues induced by adrenalectomy or hypophysectomy is accompanied by an elevation in respiratory activity. This relation does not hold in animals injected with epinephrine.

4. Thymic weights, while increased by adrenalectomy are decreased considerably by hypophysectomy. Alterations in the glycogen reserves caused by epinephrine in the lymph nodes and thymus cannot be correlated to the changes in weight undergone by these organs in the 3 groups of operated animals.

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EFFECTS OF ALTERED THYROID FUNCTION UPON THE METABOLISM OF ENDOGENOUSLY LABELED THYROID HORMONE

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THE existence of a massive enterohepatic circulation of I^{131} following administration of radioactive l-thyroxine in the rat was demonstrated in a previous paper (Albert and Keating, 1952). In the present report, the proportion of endogenously I^{131} labeled thyroid hormone in the thyroid, carcass, gastrointestinal tract and liver, and the rates of movement of labeled hormone in these compartments and in the excreta were determined under conditions of experimentally altered thyroid function.

MATERIAL AND METHODS

A total of 270 male weanling rats of the Sprague-Dawley strain was used. The animals were placed on a low iodine diet (Randall and Albert, 1951) for two to three weeks. After this pretreatment period, each rat was injected subcutaneously with carrier-free I^{131} (from 1 to 10 microcuries) and the animals were maintained on the low iodine diet from four to six days. At the end of this time, virtually all of the inorganic radioiodide administered to the animal had been either collected by the thyroid or else excreted in the urine and feces. Therefore, essentially all of the I^{131} present in the body of the rat four to six days after administration had passed through the thyroid and represented labeled thyroid hormone or radioactive metabolites of it. Furthermore, a steady state with respect to all I^{131} present in the body with body I^{131} in equilibrium with thyroidal I^{131} may be presumed, since no redistribution of I^{131} in the body subsequently occurs despite the fact that I^{131} is continually lost from the body via urine and feces.

Animals so prepared were segregated into groups and given various substances to alter the rate of secretion of thyroidal I^{131} (Albert and Tenney, 1951). In the first experiment (Table 1), groups of animals were maintained on the low iodine diet as controls, and other groups were given thiouracil (0.1 per cent in the diet), thyroglobulin (10 micrograms of thyroxine) or thyrotropin (5 mg.) by daily parenteral injection.[1] In one series animals were killed at two days (group B, Table 1), in another, at five days (group C) and in a third at zero, three, seven and eleven days (group A) after exposure of the groups to the various experimental agents. The animals were dissected into three portions: (1) the thyroid gland, removed in block fashion by removal of the trachea and larynx;

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[1] Thyrotropin used was kindly supplied by The Armour Laboratories, Chicago, Illinois, and the soluble thyroglobulin by the Chilcott Laboratories, Morris Plains, New Jersey.

TABLE 1. EFFECT OF VARIOUS AGENTS ON THE PROPORTIONS OF THYROIDAL AND EXTRATHYROIDAL I^{131}

Experimental details				Per cent body I^{131} in:			Per cent of controls		
Condition	Days	Group	Rats	Thyroid	Liver and gastrointestinal tract	Carcass	Thyroid	Liver and gastrointestinal tract	Carcass
Controls	0	A	8	78.0	5.5	16.5	100	100	100
	2	B	8	74.6	12.2	13.2	100	100	100
	3	A	4	77.7	7.2	15.1	100	100	100
	5	C	20	62.0	7.0	31.0	100	100	100
	7	A	4	77.0	4.9	18.1	100	100	100
	11	A	3	66.2	6.6	27.2	100	100	100
Thiouracil	2	B	4	62.8	16.6	20.6	84	136	156
	3	A	4	49.3	22.4	28.3	63*	311*	187*
	5	C	10	41.4	18.5	40.1	67*	264*	129
	7	A	3	13.0	21.0	66.0	17*	427*	365*
	11	A	4	8.1	10.2	81.7	12*	156	300*
Thyroglobulin	2	B	6	89.4	2.3	8.3	120*	19*	63
	3	A	4	82.6	2.4	15.0	105	33*	97
	5	C	10	83.7	1.0	15.3	135*	14*	49*
	7	A	4	84.4	0.2	15.4	110	5*	85
	11	A	3	83.9	3.2	12.9	127*	49*	47*
Thyrotropin	2	B	4	62.0	14.6	23.4	83*	120	177*
	3	A	4	53.6	18.5	27.9	69*	257*	184*
	5	C	10	60.5	4.4	35.1	98	63	113
	7	A	4	50.1	5.2	44.7	65*	106	246*
	11	A	4	50.8	8.7	40.5	77*	133	148

* Significant for $p<0.05$.

(2) the liver, stomach, small and large bowel, removed in another block dissection, and hereafter referred to as gastrointestinal tract; (3) the remainder of the carcass. The I^{131} present in each of these three portions of the body was determined by methods previously described (Johnson and Albert, 1951) and the percentage of the total body count comprising each of these three portions was calculated.

In the second experiment (Table 2), rats were injected for six days with 1 microcurie of I^{131} daily. Two days after the last injection, the same experimental maneuvers were repeated, and in addition the effects of a high iodine diet and of hypophysectomy were studied.

In the third experiment (Table 3), rats were given 1 to 5 microcuries of I^{131} in a single injection after the usual pretreatment period on low iodine diet. On the third to fifth day after injection, the rats were placed in individual metabolic cages for collection of urine and feces[2] (Albert and Keating, 1952). Groups of animals were maintained as controls, and others were given thiouracil, thyrotropin or thyroglobulin, or were subjected to hypophysectomy. Before administration of the drugs, the total body I^{131} was determined

[2] Urine and feces were determined separately. Generally, the urinary excretion of I^{131} was about twice that of the feces, although occasionally, the amounts of I^{131} were the same, or the amount in the feces was greater than that in the urine. In any case, the pattern of excretion of I^{131} after thyroids were labeled endogenously was different from that following administration of radio-1-thyroxine.

and an *in-vivo* count was taken over the thyroid area (Albert, 1951). The animals were then treated with the materials and daily counts were obtained over the thyroid, for the entire rat, and for the urine and feces. The experiment was continued for one to two weeks, at the end of which the animals were killed and the proportions of total I^{131} present in the thyroid, carcass and gastrointestinal tract were determined.

The disappearance rate of thyroidal I^{131} was calculated as previously described (Albert, 1951). The disappearance rate of I^{131} of the entire rat was calculated in similar fashion, that is, the daily counts suitably corrected for physical decay were plotted on semilog paper and the proportional rate of loss was determined. The rate of excretion of I^{131} in the urine and feces was determined as follows. The daily counts of the urine and feces were corrected for physical decay to zero time (start of experiment). The sum of these counts and the count of the entire rat at necropsy corrected to zero time was taken as the total radiation at the start of the experiment. These values agreed well with the actual count of the entire rat at zero time. The percentage of this "dose" present in the daily samples of urine and feces was determined, and added accumulatively. These values were subtracted from 100%, and the differences were plotted on semilog paper. From the lines obtained, the proportional rate of excretion of I^{131} was calculated as described for thyroidal and body I^{131}.

RESULTS

The results of the first experiment are shown in Table 1. In group C, 50 animals were studied after being exposed to the agents for 5 days. In group B, 22 animals were similarly studied at the end of 2 days, and in group A, 53 animals were studied at the end of 0, 3, 7 and 11 days. In the control animals, about two thirds to three quarters of the total I^{131} in the rat was present in the thyroid, from 5 to 12 per cent in the gastrointestinal tract, and from 13 to 31 per cent in the carcass. After exposure to thiouracil, the proportion of I^{131} in the thyroid decreased rapidly; by the eleventh day, only 8 per cent of the total I^{131} was present in the thyroid. Simultaneously, the proportion of I^{131} in the gastrointestinal tract and the carcass increased. The increase in carcass I^{131} correlated with the decrease in the thyroidal I^{131}. While the proportion of the gastrointestinal I^{131} was higher than that of controls, it did not increase concomitantly with that of the carcass, or with the decline of thyroidal I^{131}. When thyroglobulin was administered, the proportion of thyroidal I^{131} was more than four fifths of the total I^{131} while the amounts in the carcass, and especially in the gastrointestinal tract, were correspondingly less. The effects of thyrotropin were similar to those of thiouracil but less marked. Only half of the total I^{131} was present in the thyroid. Percentages in the carcass and gastrointestinal tract increased but less so than with thiouracil.

The data obtained in the first experiment can be compared best by treating each of the control groups as 100% and calculating deviations in the comparable experimental groups from this level. Such treatment of the data was deemed advisable when the variability among animals prepared with I^{131} was investigated. For any group of animals maintained as a unit

on a low iodine diet, the thyroidal uptake of I^{131} as measured in terms of per cent of dose administered, varied among individuals more than 100% above and below the mean value. Thus, a mean uptake of 20% of the dose will show a range from less than 10% of the dose to more than 40% of the dose. Similarly, the total body radioactivity of rats determined at the end of 3 or 4 days in terms of the per cent of the dose administered will likewise vary 100% above and below the mean value. However, the proportions of total I^{131} shared by the thyroid and extrathyroidal tissues were exceedingly constant, in spite of rather large differences among animals in terms of per cent of dose in thyroid or body. Thus, in a group of 20 animals the values for the mean and standard error of I^{131} present in the thyroid, gastrointestinal tract and carcass were, respectively, $66.05 \pm 1.92\%$, $7.57 \pm 0.52\%$, and $25.03 \pm 1.42\%$. The high uniformity of the data is indicated by the quotients of the means and standard errors. These were, respectively, 34, 15 and 18. Different groups of animals placed on a similar diet at different times will, however, show variation as is evident among the control values shown in Table 1.

From the standard errors of the data shown in Table 1, it was possible to calculate how much of a difference in the experimental groups, expressed as per cent of control, was significant for a P value of less than 0.05. For rats exposed to either thiouracil or thyrotropin, a value of 83% of the controls and for those exposed to thyroglobulin, a value of 113% of the controls represented significant differences for the thyroidal I^{131}. For the gastrointestinal tract, a value of 173% and a value of 189% of controls were significant for the animals given thyrotropin and thiouracil, respectively. The similar value was 59% of the controls in animals receiving thyroglobulin. For the carcass, a value of 162% was significantly different for both the thyrotropin and thiouracil treated animals and a value of 62% of the controls for the animals given thyroglobulin. In general it will be seen (Table 1) that most of the changes were significant.

In the second experiment (Table 2), groups of rats were maintained on a low iodine diet and the effect of thyrotropin and thyroglobulin was reinvestigated. Another group of animals was changed after labeling with I^{131} to a diet high in iodine (*Friskies* and tap water). Some of these animals on the high iodine diet were maintained as controls, others were given thiouracil, and still others were hypophysectomized. It will be noted that essentially the same results as described in Table 1 were obtained. High iodine diet did not materially affect the proportions of I^{131} found in the three sites, the values being the same as those noted on a low iodine diet. Furthermore, hypophysectomy produced an effect similar to that induced by thyroglobulin.

Table 3 presents data obtained in three experiments in which rates of

TABLE 2. EFFECTS OF VARIOUS AGENTS ON THE PROPORTIONS* OF THYROIDAL AND EXTRATHYROIDAL I^{131} UNDER LOW AND HIGH I^{127} DIETS

Experimental details				Per cent of body I^{131} in:			Per cent of controls		
Diet	Conditions	Days	Rats	Thyroid	Liver and gastro-intestinal tract	Carcass	Thyroid	Liver and gastro-intestinal tract	Carcass
Low iodine	Controls	0	9	74.5	7.5	18.0			
		6	9	72.2	7.3	20.5			
	Average			73.3	7.4	19.3	100	100	100
	Thyrotropin	4	8	61.9	12.5	25.6	84	169	133
	Thyroglobulin	5	8	88.1	0.7	11.2	120	10	58
High iodine	Controls	5	8	71.6	6.4	22.0	98	86	114
	Thiouracil	4	8	58.3	14.7	27.0	80	199	140
	Hypophysectomy	12	5	91.3	1.4	7.3	125	19	38

* Confidence limits are similar to those of Table 1.

movement of I^{131} were determined. It is apparent from inspection of the control groups that the loss of thyroidal I^{131}, loss of I^{131} from the entire rat, or loss of I^{131} in excreta are of the same order of magnitude and indicate a state of equilibrium. In the first experiment (group D), thyroglobulin was administered daily for a period of two weeks. Net secretion of the thyroid was inhibited to about one fifth of the control value. The rate of loss of I^{131} from the body, and the rate of excretion were similarly diminished from control values.

When net thyroidal secretion of I^{131} was increased by thiouracil, the net loss from thyroid was twice that from the body or that in the excreta (group E). This experiment was repeated with large numbers of rats (group F). Urine and feces collections were omitted since it was now clear that the same information was obtained whether the entire rat was counted or whether the urine and feces were counted. The results showed again that the loss from the thyroid was twice as great as the loss from the entire rat.

The proportions of I^{131} present in the thyroid, gastrointestinal tract and carcass at the end of the experiment show the marked shift in labeled

TABLE 3. INFLUENCE OF THIOURACIL AND THYROGLOBULIN ON THE LOSS OF I^{131} FROM THE THYROID AND BODY OF RATS

Experimental details			Rates of disappearance of I^{131}, per cent per day			Percentages of body I^{131} in:		
Group	Condition	Rats	Thyroid	Body	Excreta	Thyroid	Liver and gastro-intestinal tract	Carcass
D	Controls	16	16.06±0.80	11.52±0.59	12.47±3.42	75.52±1.34	4.89±0.34	19.64±1.13
	Thyroglobulin	16	3.70±0.22	3.59±0.26	4.16±0.20	92.79±0.45	1.19±0.10	5.72±0.33
E	Controls	4	16.83±0.58	14.82±1.24	11.26±0.57	73.04±1.80	5.78±0.41	21.18±1.84
	Thiouracil	4	60.22±3.35	32.81±1.81	32.72±1.13	4.76±1.30	19.70±1.19	75.56±0.73
F	Controls	16	9.87±0.63	6.98±0.49				
	Thiouracil	13	77.55±6.67	39.23±1.85				

hormone evoked by thyroglobulin and thiouracil. These data are in agreement with those previously described.

DISCUSSION

The experiments in this paper were designed to describe the metabolism of endogenously labeled thyroid hormone. In the period of two weeks in these experiments, the proportion of thyroidal I^{131} remained fairly constant in the control animals, ranging from 2/3 to 3/4 of the total body I^{131}. This proportion was not affected by either a low or high iodine diet. Agents, such as thiouracil or thyrotropin, which intensified the release of labeled hormone produced a marked reduction in the proportion of thyroidal I^{131}. In the case of thiouracil, the reduction increased with the duration of time the animals were exposed to it and was more severe than the reduction achieved by thyrotropin. Whereas under the influence of thiouracil the proportion of thyroidal I^{131} was reduced to 1/10 of the total radiation, the decrease produced by thyrotropin seemed not to go beyond 1/2 that of the total radiation. Among the explanations for this difference is the likely possibility that the re-accumulation in the thyroid of labeled iodide liberated from the metabolism of labeled hormone was intensified by thyrotropin and prevented by thiouracil. Among agents which caused retention of labeled hormone, the procedure of hypophysectomy and the administration of thyroglobulin induced about equal effect. In the case of the latter, a retention of labeled hormone so that the proportion of thyroidal I^{131} was from 4/5 to 9/10 of the body I^{131} occurred rapidly and did not seem to be markedly intensified with the passage of time.

The extrathyroidal I^{131} of course changed inversely with the foregoing changes in thyroidal I^{131}. The average value of the extrathyroidal I^{131} in the control rats was 27% of the total I^{131}. This value was not changed by substitution of the high for the low iodine diet, but was increased twofold to threefold by thiouracil, and about twofold by thyrotropin. It was decreased by 1/2 after thyroglobulin therapy or after hypophysectomy. These changes in the extrathyroidal I^{131} were reflected in both of its components, the gastrointestinal tract and the carcass. When the extrathyroidal I^{131} was increased, the increase was shared about equally between the gastrointestinal tract and carcass; that is, 1.5 to 4 times control values for thiouracil, and 1 to 2 times control values for thyrotropin. However, when the extrathyroidal I^{131} was decreased, as under thyroglobulin therapy, there was a disproportionate change. A greater decrease as compared with control values occurred in the gastrointestinal tract than in the carcass.

This interesting behavior of the gastrointestinal tract under opposing conditions of secretion of labeled thyroid hormone was borne out by another type of calculation. The extrathyroidal I^{131} was considered in each

case to represent 100% and the proportions in the gastrointestinal tract and the carcass were calculated. These values were then averaged for a group as a whole irrespective of the duration of treatment with any of the agents. The mean value for the 65 control animals (Tables 1 and 2, low iodine diet) was 27 and 73%, respectively, for the proportions contributed by the gastrointestinal tract and the carcass. When animals were maintained on a high iodine diet the values were unchanged (23 and 77%, respectively). For animals fed thiouracil, the proportions were 33 and 67% and for thyrotropin, 28 and 72%. These values also do not appear to differ markedly from those of the control animals. They indicate that, while the organism was being flooded with labeled hormone, the proportion of hormone in both extrathyroidal areas was not very different from the proportion of hormone during normal secretion. However, the values obtained when the labeled hormone was retained in the thyroid seem to imply a different set of circumstances. For thyroglobulin, similar mean values of all of the groups were 9 and 91% and for hypophysectomized rats 15 and 85%. Thus, under conditions of minimal liberation of labeled hormone, there was disproportionately less present in the gastrointestinal tract than in the carcass. The exact meaning of this finding is not clear. Both the hypophysectomized and the thyroglobulin-treated animals have in common a reduced liberation of thyroidal I^{131}. However, the former may be regarded as potentially hypothyroid animals, whereas the latter are hyperthyroid owing to the exogenous thyroidal therapy.

The extensive alterations induced in the proportions of total I^{131} would indicate that corresponding alterations would be induced in the excretion of I^{131} from the body. Indeed, fluctuations in excretion corresponding to the alterations in proportions were found by measuring the rate of loss of I^{131} from the thyroid, from the body, and into the excreta. Furthermore, observations on the movement of I^{131} from thyroid, body and excreta indicate a state of equilibrium for normal rats and for rats given thyroglobulin. A state of equilibrium appears not to exist for rats given thiouracil since the proportional loss of thyroidal I^{131} was much greater than the proportional loss of I^{131} in the excreta, or from the body of the rats. It may be inferred that the ability of the body to metabolize and excrete labeled hormone is fixed at some upper limit. Within this limit, the rate of thyroidal secretion may determine or at least equal the rate of metabolism; but above this upper limit, the rate of metabolism of labeled thyroid hormone is independent of the rate at which thyroid hormone is delivered to the body. That the rates of metabolism of endogenously labeled thyroid hormone are independent of the total pool of thyroid hormone in the body is indicated by finding that hypophysectomized rats showed the same reduction in rates

of movement as did thyroglobulin-treated rats. The former animals should have less total thyroid hormone than the latter.

The fact that the same effects on proportions and proportional rates of movement of labeled hormonal I^{131} were obtained despite differences in total hormone pool makes it difficult to characterize these results in terms of absolute quantities and absolute rates. Thus, in the absence of actual data on the total hormonal pool and the proportions of the pool present in the three compartments studied, the present results are intelligible only if limited to the behavior of I^{131}. Even within this limitation, the results are meaningful only to the extent that during the time of study the I^{131} labeled hormone is in a steady state.

One indication of the existence of a steady state is the constancy of the proportions of total I^{131} present in the thyroid, gastrointestinal tract, and carcass. In the period of time during which these observations were made, a period of about 2 weeks, the proportions of I^{131} in these 3 regions of the body remained fairly constant despite the fact that the total amount of I^{131} in the rats had decreased to about 1/10. Such a constancy would indicate that a steady state, at least for the major rapid phases of thyroid hormone metabolism, was present, but does not preclude the existence of slow, and therefore hidden, components. To prolong the period of time during which equilibration of labeled thyroid hormone could occur, the animals in the second experiment were injected daily for six days with I^{131}. The same proportions of total I^{131} were observed in these animals as in those given a single dose of radioiodide. Thus, no indication of the existence of a slow component was uncovered, although from the time relationships involved, it can only be concluded that, were a slow component present, the experimental maneuver was not sufficiently prolonged to expose it.

While it seems clear that all of the I^{131} of the rat is in equilibrium, it does not follow necessarily that the I^{131} is in equilibrium with the total unlabeled hormonal pool. Were this actually the case, then the ratio of the per cent I^{131} to per cent I^{127} in the three portions of the rat would be unity assuming that the I^{127} content of any tissue would represent predominantly the iodine (I^{127}) of thyroid hormone. Homogenates were made of the thyroid, the gastrointestinal tract and carcass. Aliquots were analyzed for both I^{131} and I^{127}, and the ratios I^{131}:I^{127} were determined. These ratios were not unity. The proportions of I^{131} in the thyroid, gastrointestinal tract, and carcass were respectively 75, 5 and 20%. The corresponding proportions of I^{127} were 2, 24 and 74%. Owing to technical difficulties of determining I^{127} in homogenates of tissue other than thyroid and in the absence of available similar data in the literature, one cannot be certain that the carcass I^{127} and gastrointestinal I^{127} were not spuriously high. If the values

for I^{127} are correct or even approximately correct[3], then one must conclude that a large pool of iodine is present in the rat; and that it has a very slow turnover. An additional conclusion is that this large pool of body iodine may be unrelated at least directly to the iodine present in thyroid hormone. A similar situation exists for the human. The thyroid is said to contain 20% of the total I^{127} of the body (Salter, 1940).[4] Calculation from the data of Rall *et al.* (1949), however, shows that the human thyroid, like that of rats contains more than 60% of the total I^{131} of the body several days after administration of I^{131}. Consequently, if these values are correct there exists in man also a large portion of iodine either unrelated to the iodine of thyroid hormone or not labeled by thyroidal I^{131} because of slow equilibration. More work is necessary to affirm or deny the suspicion that a hitherto unsuspected large pool of organic iodine exists in the rat, and if so, to determine its relation to the metabolic circuit of iodine related to thyroid hormone. It is clear, however, that at present, the results of this study can be discussed only in terms of labeled thyroid hormone, and that these data cannot be applied to the behavior of total body iodine.

SUMMARY

The thyroids of rats were labeled with I^{131}, and then exposed to agents or procedures which caused either liberation or retention of labeled hormone. The proportions of I^{131} in the thyroid, gastrointestinal tract and carcass were determined. Thiouracil caused an intense loss of thyroidal I^{131} and a symmetrical increase in I^{131} of the gastrointestinal tract and carcass. Thyrotropin induced similar but less intense loss of thyroidal I^{131} and a symmetrical gain in I^{131} of the carcass and gastrointestinal tract. Hypophysectomy, or therapy with thyroglobulin, produced a marked increase in thyroidal I^{131} and a marked and asymmetrical decrease in the extrathyrcidal I^{131} with a greater decrease occurring in the gastrointestinal tract than in the carcass.

There is a wide range over which the excretion of I^{131} from the body is proportional and in equilibrium with the secretion or loss of I^{131} from the thyroid. When liberation of thyroidal I^{131} is inhibited by therapy with thyroglobulin, the excretion of I^{131} in feces and urine is correspondingly inhibited. However, when liberation of thyroidal I^{131} is accelerated by thiouracil, the fecal and urinary excretion of I^{131} is also accelerated but does not keep pace with the liberation of I^{131} from the thyroid. There appears to be a ceiling beyond which the body cannot further excrete labeled thyroid hormone.

[3] Preliminary determination of I^{127} by neutron activation supports the chemical determination of I^{127}.

[4] For other estimates of the total I^{127} of the body, the reader is referred to Riggs (1952).

The metabolic behavior of I^{131} in rats labeled with I^{131} cannot be applied at present to all of the I^{127} of the body. The I^{131} excreted in the urine and feces, or present throughout the tissues of the body, is in equilibrium with I^{131} of the thyroid, but not with the total I^{127} present in the rat.

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THE RESPONSE OF THE PLASMA PROTEIN-BOUND IODINE OF HYPOPHYSECTOMIZED DOGS TO INJECTED THYROTROPIN: THE INFLUENCE OF CORTISONE

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THE thyrotropic hormone of the adenohypophysis regulates the ability of the thyroid to concentrate iodide from plasma (Rawson, 1949; Vanderlaan and Greer, 1950), to synthesize thyroxine (Chaikoff and Taurog, 1949), and to discharge thyroid hormone into plasma (Vanderlaan and Greer, 1950). It is well known that hypophysectomy results in a decrease in plasma protein-bound iodine concentrations (Taurog, Chaikoff, and Bennett, 1946). Since plasma protein-bound iodine concentrations are proportional to plasma thyroxine concentrations (Bassett, Coons, and Salter, 1941; Taurog and Chaikoff, 1948), variations in plasma protein-bound iodine reflect the status of circulating thyroid hormone. It is the purpose of this report to present the changes in the plasma protein-bound iodine concentrations due to hypophysectomy and to thyrotropin injection in hypophysectomized dogs.

A number of reports have appeared recently which indicate that cortisone and ACTH depress thyroid function, as measured by radioactive iodide uptake, in the human (Hill, Reiss, Forsham, and Thorn, 1950; Wolfson, Beierwaltis, Robinson, Duff, Jones and Eya, 1950; Berson and Yalow, 1952; Frederickson, Forsham, and Thorn, 1952) and in the rat (Perry, 1951; Verzár and Vidovič, 1951). A decrease in serum or plasma protein-bound iodine concentration has also been reported to occur in man (Hill, *et al.*, 1950; Wolfson, *et al.*, 1950; Frederickson, *et al.*, 1952; and Hardy, Riegel and Erisman, 1950) and in rats (Bondy, 1952; Foster, 1952) during cortisone therapy. The influence of cortisone on the ability of injected thyrotropin to increase plasma protein-bound iodine concentrations in hypophysectomized dogs was investigated in an attempt to ascertain the effect of cortisone on this aspect of thyroid function.

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MATERIALS AND METHODS

Healthy young mongrel dogs of both sexes were used. Their diet was Purina dog chow containing 0.5% iodized salt, with supplemental ration of fresh horse meat. Hypophysectomies were done via the oral approach. The anesthetic was intravenous sodium pentobarbital.

Plasma protein-bound iodine determinations were done by a modification (O'Neal and Simms, 1953) of the chloric acid method of Zak, *et al.* (1952). The protein-bound iodine concentrations are expressed in micrograms per 100 ml. of plasma (μg.%). Thyrotropin[2] dosages are expressed in milligrams equivalent to USP Reference Substance. All thyrotropin injections were given subcutaneously. Cortisone acetate[3] was administered intramuscularly in all instances. The plan of the experiments employing these substances will be detailed in the description of results.

RESULTS

I. *The plasma protein-bound iodine of normal dogs*

The mean plasma protein-bound iodine of 26 normal dogs was 2.6 μg.% with a Standard Deviation of ±1.18μg.%.

II. *The fall of plasma protein-bound iodine after hypophysectomy*

Serial plasma protein-bound iodine determinations were done following hypophysectomy in dogs. The results are shown in Table 1.

TABLE 1. THE FALL IN PLASMA PROTEIN-BOUND IODINE CONCENTRATION FOLLOWING HYPOPHYSECTOMY

Dog	Wt. in kg.	Preop. PBI, μg%	Days postoperative								
			1	2	3	4	5	6	7	8	8+
K71	7.7	6.6		5.8		1.9			0.6		1.0
K74	12.7	2.3		1.1			0.5				1.0
K76	8.2	2.2	2.2		1.8		1.2				died
K77	5.5	2.8	2.8		1.7		1.0				died
K79	8.9	3.1		1.6			1.4				1.1
K81	22.0	2.7			2.0			2.1			0.5
K82	11.4	2.1				1.5				1.2	1.2
K83	10.0	5.3				4.2					3.2
K89	7.3	2.1			1.3				1.5		1.4
K90	9.3	2.1			1.8				1.3		1.2
K94	10.4	—				2.8				1.5	0.8
K95	9.1	2.0					0.6				died
K96	8.6	—			1.0		1.3				died
K97	7.3	2.0			1.6		0.7				0.4
K99	11.8	2.4					1.2				—
K100	14.1	—					1.2				0.8
Mean ± S.E.$_m$		2.9 ± 0.39			1.6 ± 0.12*		1.0 ± .10*				1.1 ± .22*

* $p = 0.01$.

Insufficient data are available for the first day after hypophysectomy. A detectable decline in plasma protein-bound iodine concentration was observed in the three dogs studied on the second day and in the seven dogs studied on the third day after operation. The low hypophysectomy level

[2] The thyrotropin was supplied by Armour & Co., Chicago, Ill. Lot #R377157.

[3] The saline suspension of cortisone acetate was supplied by Merck and Co., Rahway, N.J.

of 1.0 μg.% was reached by the fifth day, after which there were no further significant changes. Dog K74 was followed for 6 months and no further change in protein-bound iodine was observed. The completeness of hypophysectomy has been confirmed by autopsy in dogs K79, 81, 83, 89 and 97. Dogs K82 and K94 showed on microscopic study of sellar contents that small remnants of adenohypophysis remained. The responses of the plasma protein-bound iodine of these two dogs were not different from those of the adequately hypophysectomized dogs. The remaining dogs were either not examined at death or are living at the time of writing. The behavior of K83 was atypical in that the protein-bound iodine concentration, initially at a high level for dogs, did not fall so rapidly as in the other dogs. Since hypophysectomy was proved to be complete in this dog by autopsy and serial sections of the contents of the sella and drill-hole, this behavior is inexplicable. After injections of thyrotropin the plasma protein-bound iodine of K83 fell to a value within the hypophysectomy range.

III. *The response of plasma protein-bound iodine to a single injection of thryotropin. The influence of cortisone on this response*

Three hypophysectomized dogs (K90, K99, K100) were each given a single subcutaneous injection of 10 mg. of thyrotropin after the plasma protein-bound iodine concentrations reached low hypophysectomy values. Serial plasma protein-bound iodine determinations were then done. After the protein-bound iodine again returned to low levels, a course of intramuscular injections of cortisone 100 mg. daily was begun. On the fourth day of cortisone therapy, another single subcutaneous injection of 10 mg. of thyrotropin was administered, and serial protein bound iodine determinations again done. Cortisone injections were continued until the plasma protein-bound iodine concentrations returned to values near 1 μg.%.

The responses of the protein-bound iodine to thyrotropin injection in these experiments are shown in Figure 1. Detectable elevation of plasma protein-bound iodine concentrations were noted as early as 4 hours after the injection of thyrotropin. Maximum levels were reached during the second day. Thereafter, the protein-bound iodine steadily declined and reached the pre-treatment low levels on the fourth or fifth day.

The data are susceptible to analysis in the following manner:

Figure 2 is a semilogarithmic plot, with protein-bound iodine concentration on the logarithmic ordinate and time on the arithmetic abscissa. The *A* curve represents the response of the plasma protein-bound iodine to the injection of thyrotropin, which was given at zero time (t_0). The points on the *A* curve are the actual plasma protein-bound iodine concentrations in μg. per 100 ml. minus the final equilibrium concentrations (PBI − PBI eq). The equilibrium values were all within the hypophysectomy range,

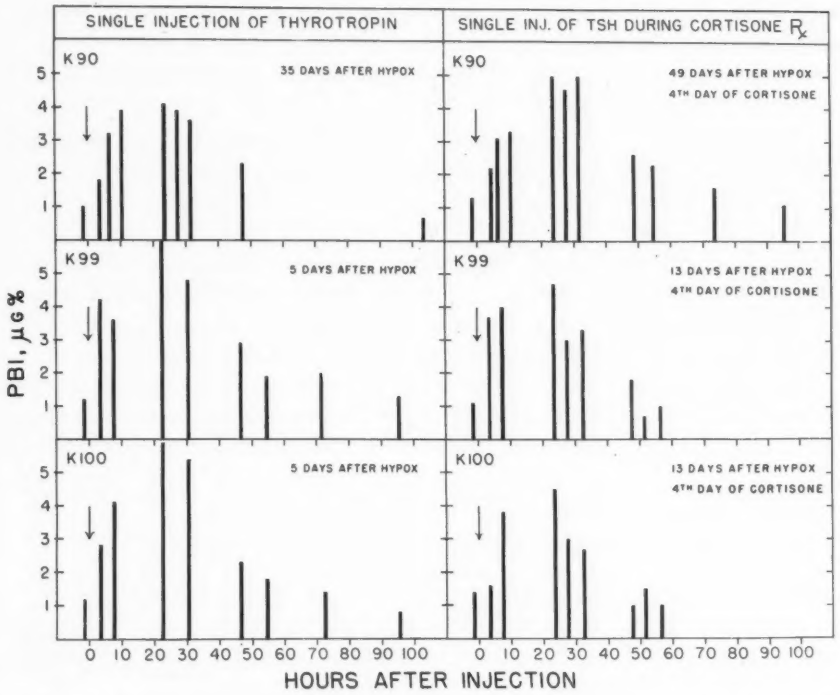

FIG. 1. The responses of the plasma protein-bound iodine concentration to a single injection of thyrotropin. On the left are the changes when thyrotropin was given alone. On the right, thyrotropin was given on the fourth day of cortisone therapy. Cortisone therapy was continued until the protein-bound iodine fell to near 1 μg%.

at or near 1 μg.%. The *A* curve is quite obviously the result of two processes: the appearance of protein-bound iodine in plasma, and disappearance of protein-bound iodine from plasma. The portion of the *A* curve which has a linear decline is believed to be due entirely to disappearance of iodine from plasma. The basis for this assumption is twofold: 1) the decline appears to be a simple exponential regression, which it would not be if further appearance of thyroid hormone were occurring, and 2) the slope of the linear decline is similar numerically to that of the disappearance of injected racemic thyroxine in thyroidectomized dogs (O'Neal, in press 1953). The decline curve is labelled *B*. The intercept of *B* at zero time (B_0) by extrapolation, is proportional to the total amount of protein-bound iodine produced by the single injection of thyrotropin, and $B_0 - B_t$ expresses the amount of protein-bound iodine which disappears from 100 ml. of plasma in t time. At any given time the concentration of *A* is the difference be-

tween the amount of protein-bound iodine which has appeared in 100 ml., of plasma and $(B_0 - B_t)$:

I. $$A_t = P_t - (B_0 - B_t)$$

P = the appearance or "production" curve. The amount of iodine which has appeared in 100 ml. of plasma at any given time is, therefore:

II. $$P_t = (B_0 - B_t) + A_t$$

The P curve represents, theoretically, the concentrations of plasma protein-bound iodine which would be expected if there were no disappearance of protein-bound iodine from plasma. The time at which production ceases (t_{Peq}) is, by definition, that time at which A first equals B.

The slope of the P curve indicates that it is the result of multiple regressions, which would be expected since its character depends on several processes going on at different rates, including the absorption of thyrotropin,

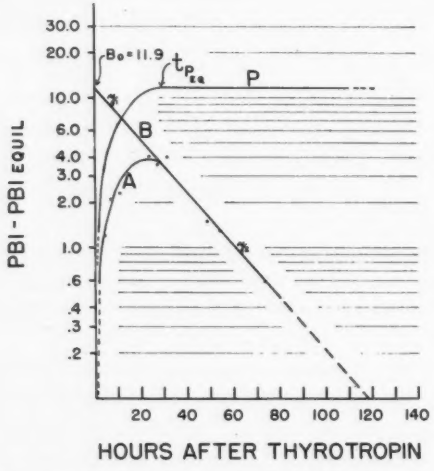

FIG. 2. Analysis of curves obtained following single injection of thyrotropin. This is a semilogarithmic plot with protein-bound iodine concentration on the logarithmic ordinate and time on the arithmetic abscissa. See text. Curve is that of dog K90 during cortisone therapy.

A = Actual plasma protein-bound iodine concentrations minus final equilibrium values ($PBI - PBI$ eq), $\mu g\%$.

B = The curve of disappearance of protein-bound iodine from plasma.

P = The curve of appearance of protein-bound iodine in plasma.

t_{Peq} = The time at the completion of accumulation of protein-bound iodine in plasma, hours.

y_1, y_2 = Points on B curve.

B_O = The intercept of the B curve at zero time by extrapolation.

the formation of thyroid hormone, the secretion of thyroid hormone, and probably other factors. The P curve is not susceptible to simple analysis with the available data. It should be noted, however that the appearance of thyroid hormone in plasma under these circumstances is a negatively accelerated process.

In separate experiments, the volume of distribution of d,l-thyroxine in dogs was determined to average 35 per cent of body weight (O'Neal, to be published). Since, for practical purposes, plasma protein-bound iodine is thyroxine iodine, an *estimate* of the total amount of thyroxine produced by a single injection of thyrotropin in these hypophysectomized dogs may be made:

III.
$$T = \frac{\left(\frac{B_0}{0.653}\right) \cdot \text{V.D.}}{100}$$

T = total thyroxine, μg.
V.D. = volume of distribution in ml. = Body wt., g. $\times 0.35$
0.653 = the fraction of thyroxine which is iodine, by wt.

In Table 2 are shown the actual and the calculated values of several aspects of the response of the plasma protein-bound iodine to single injections of 10 mg. of thyrotropin in hypophysectomized dogs. The nor-

TABLE 2. RESULTS OF SINGLE INJECTIONS OF THYROTROPIN IN HYPOPHYSECTOMIZED DOGS

Dog	Wt., kg.	Treatment	Max	B_0	T	t_{Peq}	b	$B\frac{1}{2}$
K90	9.3	10 mg. TSH	4.1	9.8	353	32 h	0.037	18.8 h
		10 mg. TSH +cortisone	5.0	11.9	428	27 h	0.040	17.3 h
K99	11.8	10 mg. TSH	6.0	12.3	778	23 h	0.040	17.3 h
		10 mg. TSH +cortisone	4.7	11.9	753	ca. 20 h	0.056	12.4 h
K100	14.1	10 mg. TSH	5.9	19.0	1436	26 h	0.057	12.2 h
		10 mg. TSH +cortisone	4.5	17.6	1332	24 h	0.071	9.8 h

Max = Maximum protein-bound iodine, actual values, μg%.
B_0 = The intercept of curve B at zero time, μg%.
T = Total amount of thyroxine produced, μg.
t_{Peq} = Time at the completion of "production," hours.
b = Slope of "disappearance" curve B.
$$= \left(\frac{\log_{10} y_2 - \log_{10} y_1}{t_2 - t_1}\right) 2.3.$$
$B\frac{1}{2}$ = Biological half-life of plasma protein-bound iodine, hours.

mal ranges of variations of the values given in Table 2 are not known. However, it appears that cortisone did not influence the magnitude or duration of the thyroid secretory response to thyrotropin injection in hypophysectomized dogs. The significance of the increased rate of disappearance or protein-bound iodine from plasma in dog K99 during cortisone therapy is not known. Comparison of the slope and of the biological half-life of the disappearance curve of K99 during cortisone therapy with the normal values of K100 suggests that the apparent increased rate of disappearance in K99 is within the limits of normal variation. Cortisone did not appear to alter the rate of disappearance of plasma protein-bound iodine in dogs K90 and K100.

IV. *The response of plasma protein-bound iodine to repeated injections of thyrotropin. The influence of cortisone on this response.*

Hypophysectomized dogs were injected with thyrotropin subcutaneously twice daily at 9:00 A.M. and at 5:00 P.M. Blood samples for protein-bound iodine analysis were drawn immediately before an injection of thyrotropin. Two dogs were given thyrotropin 5 mg. twice daily for 7 and 12 days. Two dogs were given thyrotropin 5 mg. twice daily beginning on the fourth day of cortisone therapy. Intramuscular injections of 100 mg. of cortisone daily were continued during the course of thyrotropin injections. Two dogs were given 5 mg. of thyrotropin twice daily for 26 days; during the second week of this therapy daily injections of 100 mg. of cortisone were given. Finally, 2 dogs were injected with 2.5 mg. of thyrotropin for 7 days, one without other treatment and the other receiving a course of cortisone therapy. Serial determinations of plasma protein-bound iodine were done. The details of therapy and the responses of the individual animals are shown in Figures 3 and 4.

As noted previously, the response to a single injection of thyrotropin was of 20–30 hours duration. The duration of the initial period in which plasma protein-bound iodine concentrations increased during the course of repeated injections of thyrotropin varied from two to five days with a mean duration near three and one half days. After reaching maximum concentrations the protein-bound iodine levels declined while injections of thyrotropin were being continued at the same dosage and frequency. After discontinuing thyrotropin injections, the protein-bound iodine rapidly (2–3 days) returned to low hypophysectomy levels. Thus there are two phases of the response of the plasma protein-bound iodine to repeated injections of thyrotropin: an initial sensitive phase during which the rate of production of thyroid hormone exceeds the rate of disappearance, and a later relatively insensitive phase during which the rate of disappearance equals or exceeds the rate of production.

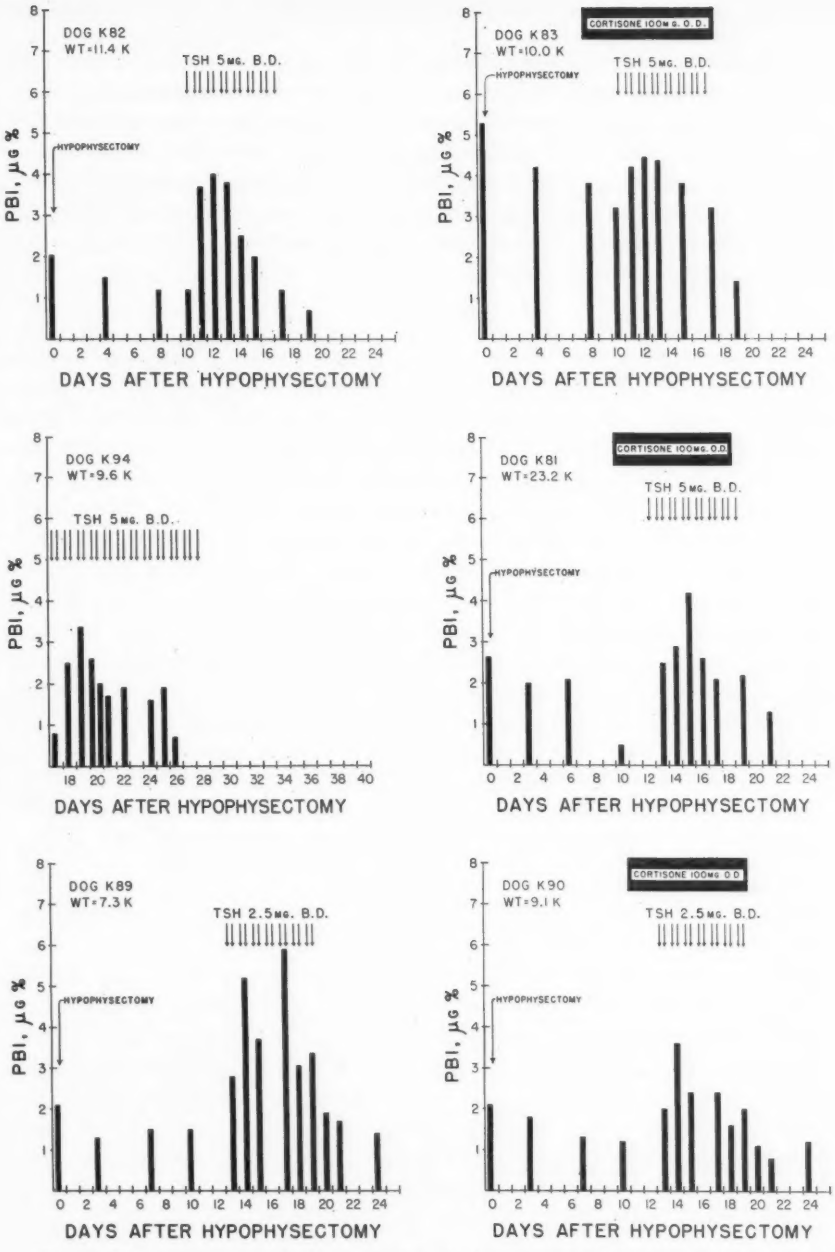

FIG. 3. The plasma protein-bound iodine during repeated injections of thyrotropin. On the left are the results with thyrotropin alone. On the right, thyrotropin was given during cortisone administration.

In Table 3 are summarized changes in plasma protein-bound iodine concentrations during repeated injections of thyrotropin. The duration of rising protein-bound iodine concentration, the maximum concentrations of protein-bound iodine, and the average protein-bound iodine concentrations during the relatively insensitive phase did not differ significantly in the dogs treated concurrently with cortisone. After cessation of injections, the protein-bound iodine returned to hypophysectomy levels with equal rapidity in all animals.

DISCUSSION

The observed decline of the plasma protein-bound iodine after hypophysectomy in dogs is similar in its time relationships to that in other species. Taurog, Chaikoff, and Bennett (1946) and Chaikoff and Taurog (1949) observed a decrease in the concentration of plasma iodine as early as two days after hypophysectomy in the rat. By four days after operation, the maximum decrease of plasma protein-bound iodine had occurred. The nature and metabolic significance of the plasma protein-bound iodine present after hypophysectomy are not clear. The actual values, with a mean of about 1 μg. per cent in hypophysectomized dogs are larger than the Standard Deviation of duplicate analyses from their mean, which in our hands was ± 0.3 μg.%. This indicates that the iodine is actually present and is not an analytical artifact. The average slope and biological half-life of the plasma protein-bound iodine disappearance curve after

TABLE 3. THE RESULTS OF REPEATED INJECTION OF THYROTROPIN IN HYPOPHYSECTOMIZED DOGS

Dog	Wt., kg.	Inj. begun, days after hypophysectomy	Pre-treatment PBI μg%	Maximum PBI μg%	Duration of rising PBI days	Average PBI, μg% in insensitive phase	Post-injection PBI μg%
Treatment: Thyrotropin 5 mg. twice daily							
K71 1st wk.	7.7Kg.	25days	1.2μg%	7.4μg%	4 days	—	—
K74 1st wk.	10.9	12	0.9	6.0	4	—	—
K82	11.4	10	1.2	4.0	2½	1.9	0.7
K94	9.6	17	0.8	3.3	3	1.8	0.7
Means			1.0	5.2	3½ days	1.8	0.7
Treatment: Thyrotropin 5 mg. twice daily, cortisone 100 mg. daily							
K71 2nd wk.	7.7	—	—	—	—	With cortisone: 1.9 without cortisone: 2.5	1.4
K74 2nd wk.	10.5	—	—	—	—	With cortisone: 2.0 without cortisone: 1.7	0.8
K81	23.2	12	0.5	4.2	3	2.3	1.3
K83	10.0	10	3.2	4.4	2½	3.5	1.4
Means			—	4.3	2–3	2.9	1.2
Treatment: Thyrotropin 2.5 mg. twice daily, with and without cortisone							
K89 (without cortisone)	7.3	13	1.4	5.9	5 days	2.7	1.4
K90 (with cortisone)	9.1	13	1.2	3.6	2 days	2.1	1.2

hypophysectomy ($b=0.016$; $B\frac{1}{2}=43$ hours) indicate that protein-bound iodine disappears more slowly after hypophysectomy than it does after the administration of bovine thyrotropin to hypophysectomized dogs (Av. $b=0.045$; Av. $B\frac{1}{2}=16.2$ hours). These differences may be due either to brief continuing action of the thyrotropin remaining in the animal after hypophysectomy or to alterations in the rate of disappearance of thyroid hormone in the postoperative state. There is considerable individual variation in the rate of fall of plasma protein-bound iodine after hypophysectomy.

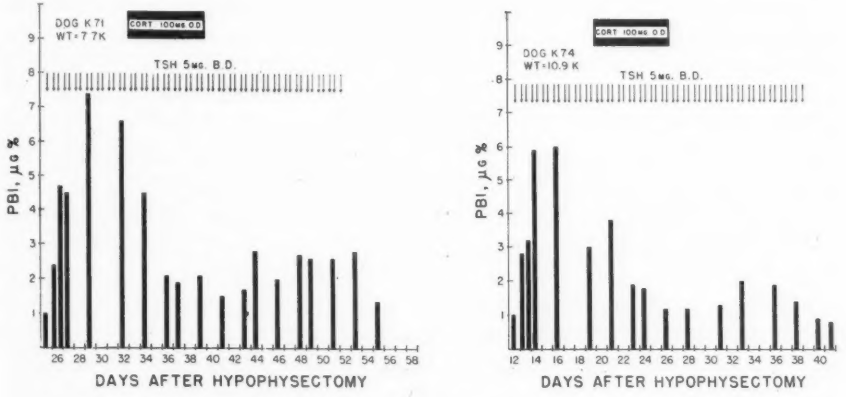

FIG. 4. The response of the plasma protein-bound iodine during prolonged courses of repeated injections of thyrotropin. Cortisone was administered during the second week of thyrotropin.

The character of the disappearance curve (B) of plasma protein-bound iodine after a single injection of thyrotropin is dependent upon the rate of destruction of organic iodine in the tissues and the rate of excretion in feces and urine. The relative part played by each of these processes in our study is not known. It is believed, however, that the decline is due chiefly to metabolic destruction of thyroid hormone, since the decline appears to follow a simple exponential regression. However, no information is available concerning losses of organic iodine by fecal and urinary excretion in dogs, and therefore "utilization" rates cannot be calculated. The short biological half-life of plasma-protein bound iodine in the dog indicates that the dog disposes of thyroid hormone at a much more rapid rate than the human does. The biological half-life of plasma protein-bound iodine averaged 16.2 hours in our experiments. Albert and Keating (1949) found the biological half-life of precipitable radioactivity in plasma after the injection of radioactive thyroxine in a cretin to be 3.8 days. Recant and Riggs (1952)

reported biological half-lives of injected racemic thyroxine to be 4.3 days in a normal subject, 4.3 days in a nephrotic subject, and 8.3 days in a patient with untreated myxedema. The short half-life of endogenously produced thyroid hormone in our experiments is in keeping with the observations of Danowski *et al.* (1946) that dogs have a greater capacity than does man to inactivate or destroy exogenous oral thyroid hormone and intravenous thyroxine.

In the dogs treated with repeated injections of thyrotropin, the plasma protein-bound iodine concentrations increased then fell, *while injections of thyrotropin were continuing.* This phenomenon is subject to at least two explanations. First it is possible that the increased concentration of thyroid hormone after injection of thyrotropin may itself accelerate the rate of metabolic destruction of thyroid hormone. Should this be the case, the result might be a fall in plasma protein-bound iodine concentrations at constant high rates of secretion. The second possibility is that the thyroid may secrete its hormone at a progressively decreasing rate during a course of repeated injections of thyrotropin; that the thyroid becomes insensitive, or relatively so, to further injections of thyrotropin. Others have observed that the initial responses to injected thyrotropin are not maintained by repeated injections of the same dosage at the same frequency. Trikojus and Ellis (1939) and Collip and Anderson (1935) found that the initial histological effects of thyrotropin injections on the thyroid of guinea pigs and of rats were not maintained and that eventually thyroid atrophy occurred. Spense and Witts (1939) reported that the initial elevation of the basal metabolic rate occasioned by thyrotropin in patients with hypopituitarism was not maintained by continued injection.

Our studies indicate that cortisone does not influence any of the functions of thyrotropin which were measured in our experiments. These include indices of the duration of increased thyroid secretion following injected thyrotropin, of the amount of thyroxine produced after a single injection of thyrotropin, of the rate of utilization of endogenously produced thyroid hormone, and of the response to repeated injections of thyrotropin. Our results are therefore consistent with Halmi's (1952) observation that the morphological effects of injected thyrotropin on the thyroid gland of hypophysectomized rats are not hampered by simultaneously administered cortisone. Woodbury, *et al.* (1951) found that both ACTH and cortisone inhibit the increase in I^{131} uptake due to exogenous thyrotropin in hypophysectomized rats. Others (Verzár and Vidovič, 1952; Brown *et al.*, 1952; Paschkis *et al.*, 1952) have confirmed this observation. Since other indices of thyrotropin action than I^{131} uptake, such as thyroid secretion rate (Perry, 1951; Albert, 1952) the ability of the thyroid to synthesize thy-

roxine (Migeon, *et al.*, 1952) and thyroid morphology (Halmi, 1952) are not altered by cortisone, it is possible that the reduced uptake of I^{131} caused by cortisone is due to extrathyroidal changes in iodine metabolism. Increased renal excretion of iodide is known to occur following cortisone therapy (Frederickson, *et al.*, 1952; Woodbury, *et al.*, 1951; Berson and Yalow, 1952; and Kuhl and Ziff, 1952).

SUMMARY

Thyroid function was studied in dogs by means of plasma protein-bound iodine determinations. The plasma protein-bound iodine concentration of normal dogs was 2.6 μg.% $\pm$ 1.18 μg.% (S.D.). Following hypophysectomy, the protein-bound iodine fell to low levels (1.0 μg.%) by the fifth postoperative day. Single injections of 10 mg. of thyrotropin in hypophysectomized dogs resulted in an increase in plasma protein-bound iodine concentration. Detectable elevation was observed at the fourth hour after injection. Maximum concentrations were reached by 24–30 hours, after which a decline to preinjection low levels occurred. Repeated injections of thyrotropin resulted in an initial elevation of plasma protein-bound iodine, followed by a decline after 2–5 days while injections of the same dosage at the same frequency were being continued. The concurrent administration of cortisone did not alter the magnitude or duration of the response of the plasma protein-bound iodine due to single or multiple injections of thyrotropin, and did not influence the rate of disappearance of protein-bound iodine from plasma following the maximum elevation after a single injection of thyrotropin in hypophysectomized dogs.

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THE ABSORPTION AND GLYCOGENIC ACTIVITY OF CORTISONE AND HYDROCORTISONE AFTER PARENTERAL ADMINISTRATION TO RATS

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REPORTS in the literature show that the free alcohols and acetic acid esters of cortisone and hydrocortisone differ in their physiological activity and therapeutic efficacy. For example, Conn, Louis, Fajans, Johnson, Barott, Hogg, Blood and Weiss (1951) reported that intramuscularly administered hydrocortisone alcohol (F Alc) produced intense metabolic effects in man, while hydrocortisone acetate (F Ac) produced only mild effects. Given orally, however, both compounds produced marked effects. Hollander, Brown, Jessar and Brown (1951) reported that F Ac was much more effective than cortisone acetate (E Ac) in the therapy of arthritis by intra-articular injections. Boland and Headly (1952) reported that, when administered orally, cortisone alcohol (E Alc) and E Ac were about equally effective in the control of arthritic symptoms, but that F Ac was less effective. F Alc was found to be about 50% more active than E Ac. Perera, Ragan and Werner (1951) have found intramuscularly administered F Ac to be less effective than E Ac in producing metabolic changes and clinical remission of arthritis in man.

Macek, Baade, Bornn and Bacher (1952) have shown that the various forms of cortisone and hydrocortisone possess different solubility characteristics, a fact which may partly explain the differences in physiological activity. An indication that differences in absorption are probably not solely responsible for differences in activity has been presented by Ingle, Nezamis and Morley (1951). These authors found that F Alc was about twice as effective as E Alc in maintaining the ability of adrenalectomized rats to do work, when the compounds were administered by continuous intravenous infusion.

The objective of the work reported here was to compare the activities and rates of absorption of cortisone and hydrocortisone and their acetates after intramuscular and subcutaneous injection. Liver glycogen deposition was chosen as a reasonably reliable, objective measure of activity. Analysis of the excised injection sites for residual hormone yielded information regarding the absorption of these substances.

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EXPERIMENTAL

Male albino rats of the Carworth strain, weighing 150–200 gm., were fasted for 48 hours, then given single doses of the steroids as microcrystalline suspensions in saline. Subcutaneous injections were administered at a single site. Intramuscular doses were divided into two equal portions, and injected into the hind legs of the rats. Immediately following the injections, and at the same time each succeeding day, the animals were fed 3 gm. of a 20% casein diet/100 gm. body weight. This quantity of food was consumed in an hour or two, so that when the rats were sacrificed 24 hours later, they had been fasted for about a day. The animals were sacrificed by means of a lethal dose of nembutal, and liver samples were hydrolyzed for the determination of total reducing substances (expressed as glucose, termed glycogen hereafter). Daily average control values ranged from 50 to 100 mg./100 gm. rat.

Discrete pockets of steroid were found under the skins of the subcutaneously injected animals. Residual deposits in the muscles were more diffuse so the entire musculature from the dorsal aspect of the hind limbs was removed for analysis. The subcutaneous deposits and the muscles were thoroughly homogenized in 80% ethanol, and the suspensions were heated to boiling and filtered. Aliquots of the filtrates were taken for the determination of steroid by the phenylhydrazine-sulfuric acid method (Porter and Silber, 1950). The reagent was added directly to the alcoholic extract.

RESULTS

Subcutaneously administered steroids. F Ac was less effective, while F Alc was more effective than E Ac in promoting the deposition of liver glycogen (Fig. 1). The greatest responses were observed 2 to 4 days after the injections, and after seven days, the liver glycogens were no longer significantly elevated.

As a measure of the total activity of the compounds, the areas under the curves relating liver glycogen and time were estimated (Table 1). Fractions of the compounds which were absorbed in seven days were estimated by analyses of the injection sites. The dependency of total activity of the steroids upon the amount of compound absorbed is indicated in the last column of the table. Comparison of F Ac with F Alc is most striking. The free alcohol was absorbed nearly quantitatively and exhibited a high degree of activity. The ester, only 20% of which was absorbed, showed a proportionately smaller effect upon liver glycogen. Although appreciable quantities (20–80%) of the doses given (except F Alc) remained under the skins after seven days, the liver glycogens of these rats were only slightly higher than those in the control animals. Apparently, the unabsorbed com-

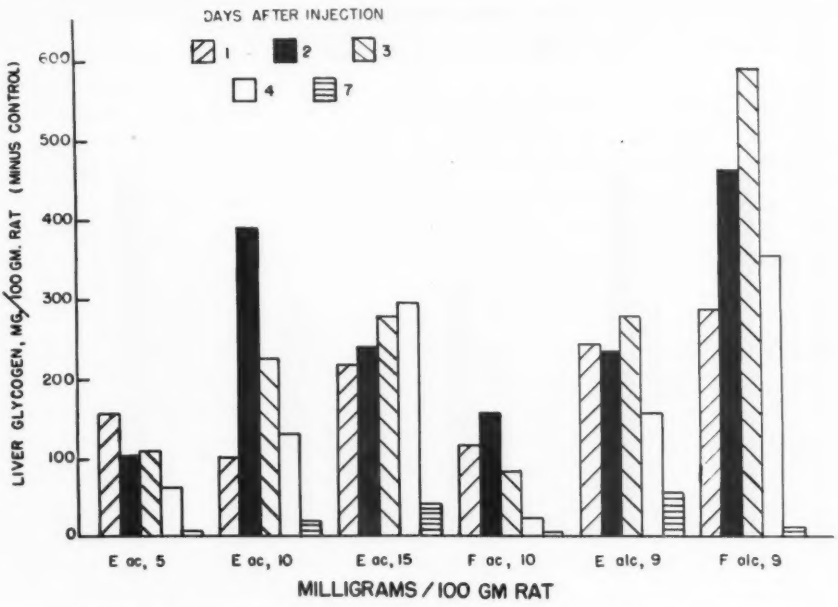

FIG. 1. Liver Glycogen after a single subcutaneous injection of steroids.

pounds were rather effectively isolated, and hence were relatively impotent after seven days. Furthermore, within the range of dosage employed, the proportion of E Ac absorbed was independent of the quantity injected.

Rats which had been injected with E Ac (15 mg./100 gm. rat) were injected again on the seventh day with half this quantity of the same steroid. Three days later, the livers of these animals contained as much glycogen as the livers of animals which had received a single injection of

TABLE 1. RELATIONSHIP BETWEEN TOTAL ACTIVITY OF STEROIDS AND AMOUNT ABSORBED FROM SUBCUTANEOUS TISSUE. FOUR RATS PER GROUP

Steroid and dose,[2] mg./100 gm. rat	Activity (7-day area)	Steroid absorbed, % of dose	Activity per mg.[1] steroid absorbed
Cortisone Acetate, 5	454	44.4	204
Cortisone Acetate, 10	995	50.7	196
Cortisone Acetate, 15	1388	49.1	190
Cortisone Alcohol, 9[2]	1148	73.7	156
Hydrocortisone Acetate, 10	396	19.3	205
Hydrocortisone Alcohol, 9[2]	2054	98.0	210

[1] Calculated as the acetate.

[2] 9 and 10 mg. doses given as 0.33 ml. suspension/100 gm. rat. 5 and 15 mg. doses, half and twice this volume. 9 mg. of the alcohols are the molecular equivalents of 10 mg. of the acetates.

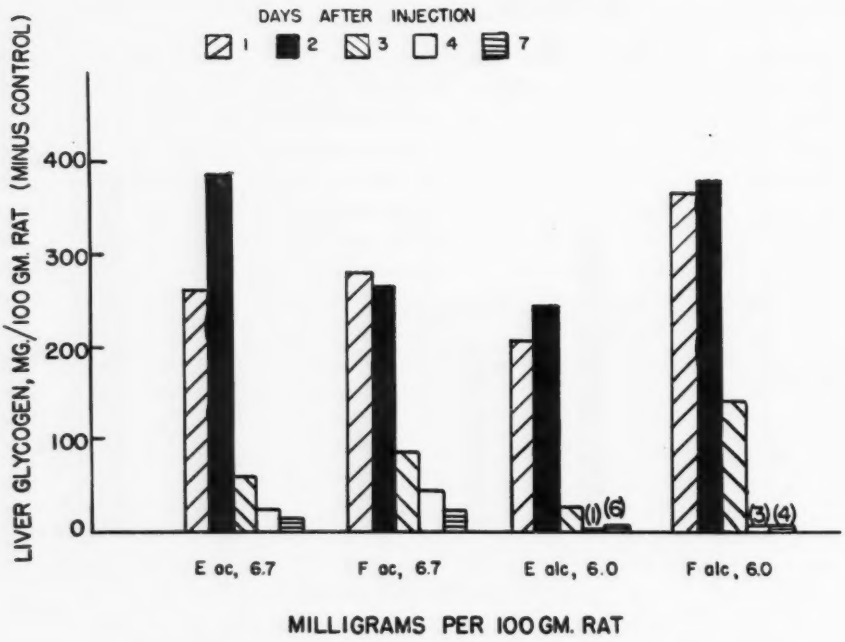

FIG. 2. Liver Glycogen after a single intramuscular dose of steroids.

E Ac (7.5 mg./100 gm. rat) three days before sacrifice. Hence, the rats did not become refractory under the conditions used. In addition, residual E Ac obtained from injection sites after seven days was found to be fully active on reinjection.

Intramuscularly administered steroids. The compounds were absorbed more rapidly and more completely from the muscle (Fig. 2, Table 2) than from under the skin. Maximal effects of the steroids were found 1–2 days

TABLE 2. RATE OF ABSORPTION OF STEROIDS AFTER INTRAMUSCULAR INJECTION. THREE RATS PER GROUP

Steroid and dose, mg./100 gm. rat[2]	Steroid absorbed, % of dose					Seven-day activity per mg.[1] absorbed
	Days after injection					
	1	2	3	4	7	
Cortisone Acetate, 6.7	77	90	98	97	—	103
Cortisone Alcohol, 6.0	73	86	100	99	—	69
Hydrocortisone Acetate, 6.7	33	62	50	50	71	177
Hydrocortisone Alcohol, 6.0	83	93	95	100	—	127

[1] Calculated as the acetate.

[2] 6.0 mg. of the alcohols are the molecular equivalents of 6.7 mg. of the acetates. The volume injected into each hind leg was 0.15 ml. per 100 gm. rat.

after the injections, and after four days the effects were minimal. F Ac was most persistent, both in effect and in quantity of steroid found at the injection site.

Although E Ac, E Alc and F Alc were absorbed at about the same rate, F Alc was superior, E Alc inferior in promoting the deposition of liver glycogen. The total activities (i.e., areas under the curves relating glycogen and time) of F Alc and E Alc were in the ratio of about 2 to 1, which agrees well with the data of Ingle, Nezamis and Morley (1951). After subcutaneous injections F Alc was found to be 1.35 times as effective as E Alc.

DISCUSSION

Hydrocortisone acetate is relatively insoluble and was slowly absorbed after parenteral administration. This property may be responsible for the reported superiority of F Ac for the treatment of arthritis by intra-articular injections, where prolonged local activity may be desired. Hydrocortisone alcohol was more rapidly absorbed, and this, combined with its apparently greater activity per unit weight, recommends F Alc as the choice of the materials studied when rapid action is desired. The results indicate that, with the exception of F Ac, the steroids were absorbed at similar rates from the muscles.

Undoubtedly, the therapeutic efficacy of steroids is governed not only by their inherent potencies, but also by the rates with which they are absorbed and destroyed. A very rapidly absorbed material might be eliminated or excreted rapidly, and therefore be less effective than a more slowly absorbed, but more efficiently utilized compound.

Assay procedures in which a steroid is injected and a physiological variable (i.e., liver glycogen) is measured are unreliable from a quantitative standpoint if all of the steroid is not absorbed, or indeed, if rates of absorption vary. It has been observed (Porter and Silber 1950)[1] that while E Ac in aqueous suspension produces a very poor liver glycogen response in a one-day test such as that of Reinecke and Kendall (1942), E Ac dissolved in propylene glycol produces a significant response. Administered daily for several days, a suspension of the same compound produces an excellent response. Even so, in view of the present work, quantitative interpretations of data derived from either procedure are of limited value unless the efficiency of absorption is determined.

The measurement of liver glycogen daily after the administration of a steroid, calculation of total activity for a one-week period, and direct measurement of absorption yields information which should constitute a more precise evaluation of the true activity of a glycogenic steroid.

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[1] Unpublished data.

SUMMARY

Of the four compounds, cortisone acetate (E Ac), cortisone alcohol (E Alc), hydrocortisone acetate (F Ac) and hydrocortisone alcohol (F Alc), F Alc was the most rapidly absorbed after intramuscular or subcutaneous injection in the rat. F Ac was the least rapidly absorbed, and in the quantity given, persisted to a high degree at the site of injection for at least 7 days.

Absorption of the compounds from muscle was more rapid than from subcutaneous tissue, as judged by chemical analysis of the injection sites and by the deposition of reducing substances (glycogen) in the livers. Subcutaneous deposits of E Ac, although relatively ineffective in promoting the deposition of liver glycogen 7 days after injection, were active when removed and reinjected.

These studies demonstrate that apparent differences in biological activity can easily result from differences in absorption.

Acknowledgment

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PRE-OVULATORY PROGESTERONE IN THE PERIPHERAL BLOOD OF THE RABBIT[1]

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THE experiments to be described were begun as an investigation of the production of progesterone by the rabbit in the earliest days of pregnancy and pseudopregnancy. Subsequently, other studies (Forbes, 1950a) having indicated that women may secrete progesterone shortly before ovulation, it was decided to search for the same phenomenon in the rabbit. This animal is well suited for such studies, since it does not ovulate spontaneously but only in response to an extrinsic stimulus, ovulation occurring 9½ to 10 hours (Hammond, 1925; Corner, 1928; Asdell, 1946) or 10 to 12 hours (Fee and Parkes, 1929) after mating. The aim of the investigation was shifted to the determination of plasma progesterone levels during the first day or two, and finally during the first hours, following the induction of ovulation by mating or by the administration of gonadotrophin (Bellerby, 1929).

A brief summary of some of the results has appeared elsewhere (Forbes, 1950b).

MATERIALS AND METHODS

Nineteen adult virgin female, New Zealand white rabbits weighing 5½ to 9 pounds each were donors. A 20th female donor (R40), weighing 11 pounds and also of this breed, had borne several litters. Ovulation was induced by mating (10 rabbits) or by injection of pituitary gonadotrophin[2] into an ear vein (9 rabbits). Occurrence of ovulation was confirmed by examination at laparotomy or autopsy of the reproductive tracts of all of the injected animals except R25, which was killed 2½ hours after injection (i.e., before ovulation could occur). Identification of free blastocysts or embryos confirmed the existence of pregnancy in 7 of 10 rabbits after mating. A postcoital smear from an 8th rabbit (R21) showed numerous motile sperm, but through error the reproductive tract was not examined later for evidence of pregnancy. A 9th rabbit (R33) was killed 8 hours after mating; her ovaries contained large follicles, but she had not yet ovulated. The 10th mated animal (R34) had a postcoital vaginal smear containing abundant motile sperm.

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[2] Pituitary Gonadotrophin Squibb was generously supplied by Messrs. H. Sidney Newcomer, T. D. Gerlough, and J. E. Aurelius of E. R. Squibb & Sons.

However, at laparotomy a week later she was not pregnant and may no longer have been pseudopregnant.

The final female (R31) of the 20 with which this report is concerned refused the male when placed in his cage; 28 minutes later she was injected with 500 R.U. of gonadotrophin.

One cc. blood samples were drawn from ear veins by syringes containing 0.05 cc. 20% sodium citrate solution and were centrifuged promptly. Measured volumes of plasma were precipitated with stirring in approximately 10 volumes of cold (4° C.) acetone. The supernatant (containing the "free" progesterone) and in some cases the precipitate (including the "bound" progesterone) were later prepared for assay according to standard procedures (Hooker and Forbes, 1949b).

The bio-assay for progesterone which was employed appears to be sensitive, accurate, and specific at least to the extent that numerous other specific, naturally occurring compounds tested have not duplicated the cytological response on which the assay is based (Hooker and Forbes, 1947, 1949a). However, since it is scarcely possible to prove completely the specificity of the assay, the term "progesterone" must be used with some reservation in connection with the results presented here. The ability of estrogen to block the cytological response to progesterone, resulting in a false negative assay, has been reported by Salhanick, Olsen, and Hisaw (1951). Hooker and Forbes (1947 *et seq.*) and Zarrow and Neher (1951) have not detected the blocking effect when dealing with what were thought to be physiological concentrations of estrogen and progesterone.

RESULTS

All assay results will be reported in μg/cc. plasma.

The data for "free" progesterone levels are presented graphically for 17 of the rabbits, μg. of progesterone/cc. plasma being plotted against time (Figs. 1–3). The symbol consisting of a circle and an arrow means that less than the indicated concentration of progesterone was present; the assay does not reveal the absence of the hormone. Successive individual assay levels on the graphs are connected by straight lines, but this is not meant to imply that intermediate concentrations of progesterone would necessarily fall on these lines. The time that mating occurred or that gonadotrophin was injected is indicated by a vertical arrow.

Control Blood Samples. In every experiment, a control blood sample was drawn just before or soon after mating or injection of gonadotrophin (Figs. 1–3). Progesterone was not detected in the control specimens from any of the 20 rabbits which were used. Samples from an additional rabbit were discarded when the control specimen showed a positive response.

"Bound" Progesterone. Assays for bound progesterone were performed on 4 to 7 samples from each of 4 rabbits chosen at random. One specimen (from R31) contained 0.5 μg. progesterone/cc. plasma; 2 (from R15) contained 0.3 μg./cc.; in the other 19 specimens, no progesterone was detected.

Day-to-Day Levels of Free Progesterone. Rabbit R9 was injected intravenously with 50 R.U. of gonadotrophin, while R10 received 125 R.U. Blood specimens were obtained on day 0 and daily thereafter for 7 days. The

specimens were drawn at approximately but not exactly the same hour each day. Whether by coincidence or otherwise, in both rabbits the blood levels of progesterone reached peaks on days 1, 5 and 7 (Fig. 1).

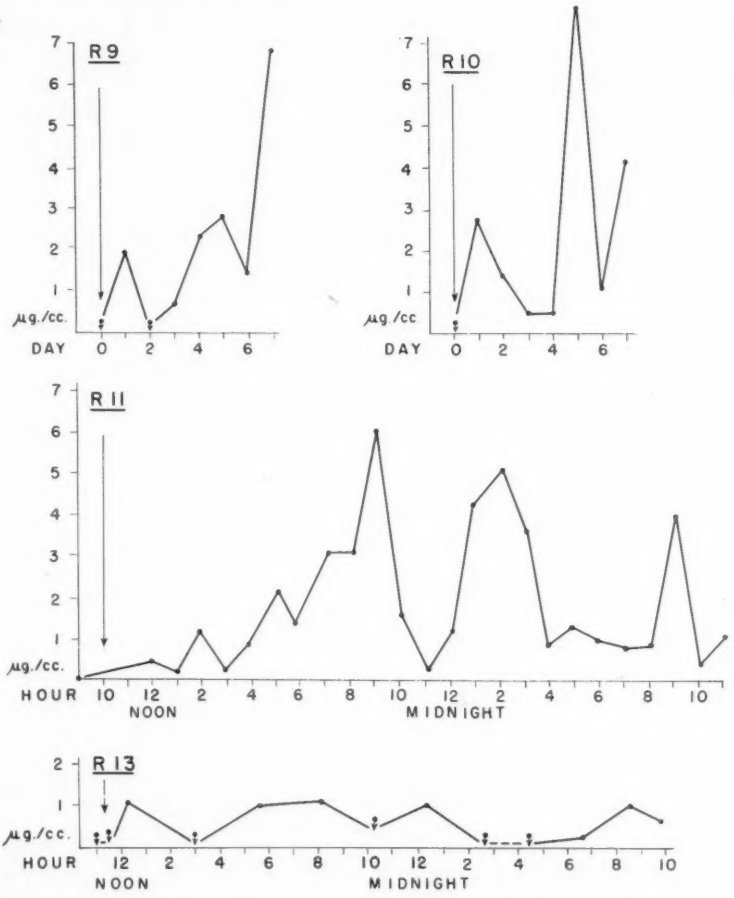

FIG. 1. Micrograms of progesterone/cc. plasma have been plotted against time in days (R9 and R10) and hours (R11 and R13). The dot and arrow symbol means that the progesterone level was less than that indicated. The large vertical arrow indicates the time when gonadotrophin was injected.

The results of these studies suggested that the time of appearance of the hormone might be determined more closely if blood samples were taken hourly for assay.

Hour-to-Hour Levels of Free Progesterone. Injections of 125 R.U. of gonad-

otrophin were made into each of 3 rabbits (R11, R12, R13) shortly after control blood samples were obtained. Additional blood samples were drawn at intervals of 1–3 hours for the next 23–24 hours. The graphs for R11 (Fig. 1) and R12 show rapid fluctuations in the plasma levels of progesterone. Peaks or high levels were detected in R11 at 4, 7, 9–11, 15–17, and 23 hours after injection (Fig. 1) and in R12 at 4, $6\frac{1}{2}$, 10–12, and $21\frac{1}{2}$ hours after injection. The maximal level from R12 of 7.4–7.5 μg./cc. at 10–12 hours after injection is comparable to the maximal level from R11 of 6.0 μg./cc. at 11 hours after injection. Samples from R13 (Fig. 1), on the other hand, never assayed higher than 1.1 μg./cc.

Progesterone was also detected in blood samples taken several hours before the expected time of ovulation from 4 mated rabbits (R14, R15, R16, R17; Fig. 2).

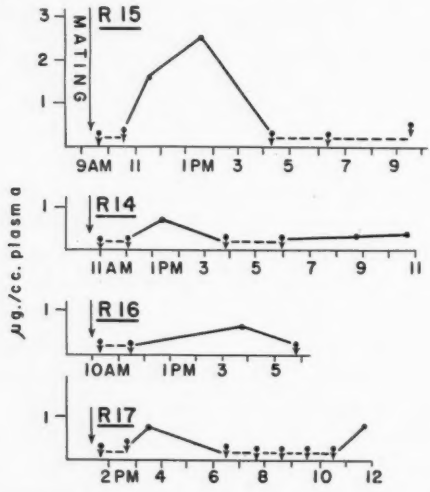

FIG. 2. The large vertical arrows indicate the time of mating.

Time of First Appearance of Free Progesterone. Blood samples were next taken at intervals of a few minutes from an additional 4 rabbits which had each been injected with 125 R.U. of gonadotrophin (R19, R22, R23, R25), from an additional 6 mated rabbits (R18, R21, R33, R34, R35, R40), and from R31 (see below). Except for the control specimens, all samples were drawn during the intervals in which it was thought progesterone might appear. Results from 9 of the 11 rabbits appear in Figure 3.

Assay data for one injected and one mated rabbit are not presented graphically since, due to faulty scheduling, the intervals between the first positive and the preceding negative specimens were 59 and 25 minutes.

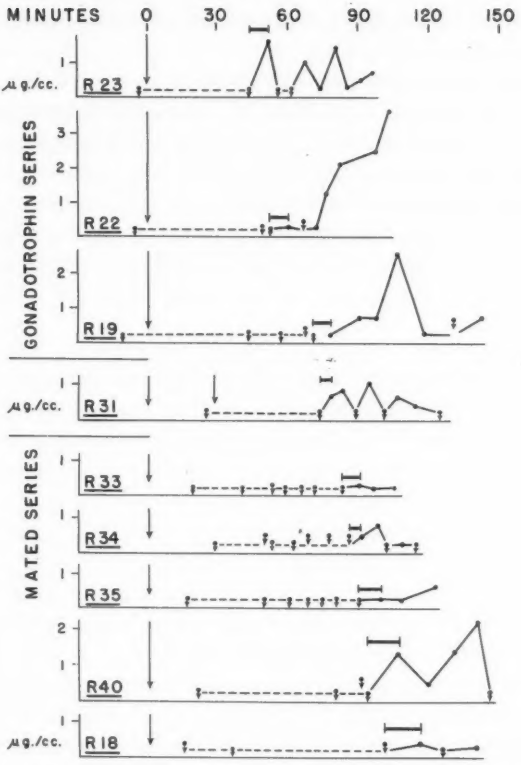

FIG. 3. The large vertical arrows indicate the time of mating or of injection of gonadotrophin. Rabbit R31 refused to mate (first arrow); 28 minutes later she was injected with gonadotrophin (second arrow). The heavy horizontal bars indicate the time interval between the first positive specimen and the preceding negative specimen; it was presumably during this interval that a detectable level of progesterone first appeared in the blood. Note that maximal blood levels are, except for R40, generally lower after mating than after injection; R40 was the only rabbit which had previously littered.

Blood samples from the other animals revealed the first appearance of progesterone 54, 60, and 78 minutes (average, 64 minutes) after injection of gonadotrophin and 90, 90, 99, 106, and 116 minutes (average, 100 minutes) after mating.

Twenty-eight minutes after R31 first refused the male's prompt and vigorous attempts to mate she was injected with 500 R.U. of gonadotrophin. Her first blood sample which was positive for progesterone was drawn 78 minutes after she was placed in the male's cage and 50 minutes after she

was injected; these intervals are shorter than those recorded for mated and injected rabbits, respectively.

Of the total of 20 rabbits from which samples were taken, 18 were bled rather soon after mating or injection. Maximal progesterone levels in samples drawn in the first 3–4 hours were lower (0.3–0.8 μg./cc.) for 8 of the mated females than were maximal levels (1.1–3.9 μg./cc.) for the 7 injected females. However, 2 other mated rabbits, R15 (virgin) and R40 (breeder), within the first 4 hours after mating yielded specimens assaying as high as 2.5 μg./cc. The maximal level for R31 (exposed to male, injected) was 1.0 μg./cc.

DISCUSSION

Blood from non-pregnant, pseudopregnant, or pregnant rabbits, mice, monkeys, and women contains little "bound" progesterone (Hooker and Forbes, 1949b; Forbes, 1950, 1951; Forbes, Hooker, and Pfeiffer, 1950a, 1950b). Results from the present experiment are in keeping with this observation.

The "free" progesterone concentrations of 6.8 and 4.1 μg./cc. measured in blood samples obtained from 2 rabbits 7 days after injection of gonadotrophin are within the range (3.6–7.0 μg./cc.) previously determined for this day of pseudopregnancy in 9 other rabbits (Hooker and Forbes, 1949b; Forbes, Hooker and Pfeiffer, 1950b).

It is now generally agreed that in the female rabbit the mating stimulus is transmitted neurohumorally to the anterior pituitary, which is thereby activated to secrete quantities of gonadotrophic hormone. The latter, in turn, stimulates the ovary to the point that ovulation occurs. Many details of this process, however, remain obscure and controversial. Temporal factors are important. As previously mentioned, 9½–10 (Hammond, 1925; Corner, 1928), or 10–12 (Fee and Parkes, 1929), hours are reported to elapse between mating and ovulation. It was demonstrated many years ago that if a female rabbit is mated, anesthetized, and hypophysectomized (either by decerebration or a less drastic procedure), ovulation would still take place unless the pituitary were removed within 1 hour post coitum (Fee and Parkes, 1929; Deanesley, Fee and Parkes, 1930; Smith and White, 1931). The gonadotrophin content of the rabbit pituitary has been shown to drop by 20% within 30 minutes after mating (Hill, 1934). In the present experiment, progesterone was detected an average of 64 minutes after administration of gonadotrophin and an average of 100 minutes after mating. This suggests that intravenous injection of gonadotrophin immediately raised the blood concentration of this hormone to the effective level which pituitary secretory activity, set off by the mating stimulus, required ½ hour to duplicate. (A dose of 125 R.U. of course may have considerably

exceeded the effective level.) The data also suggest that when an adequate amount of gonadotrophin is delivered all at once to the organism, the cells which are to secrete the first progesterone still require an hour before they can produce detectable amounts of that hormone.

It is perhaps more than coincidence that in both of the rabbits (R9 and R10) from which samples were obtained at daily intervals, peak blood levels occurred on days 1, 5, and 7. On the other hand, except for rabbits R11 and R12, the graphs for blood samples taken at intervals of 1–3 hours show little similarity. Apparently one is not likely to find a regular secretory pattern during the first day or two of progesterone production in the rabbit.

The source of progesterone in the absence of functional corpora lutea is uncertain. Production of the hormone by the adrenal cortex has been reported. It may be that scattered cells in the wall of the follicle can rapidly assume a luteal function following adequate stimulation. In 1919 Corner noted that some of the theca interna cells of the ripe follicle of the sow contained vacuoles of fat-like material. Seaborn (1935) observed luteal cells in the granulosa and theca interna of the ripe follicle of the mare; sometimes such cells were present on the first day of estrus. Wimsatt (1944) observed luteinization of the pre-ovulatory follicular cells of the bat, and suggested that the progestational development of the uterus shortly before ovulation indicated that progesterone had already been secreted. The granulosa and theca interna of the human follicle just prior to, during, and soon after ovulation are reported to contain numerous and distinctive "K cells"; such cells "represent the site of intense localization of ketonic lipid, if not the site of production or utilization of ketonic lipids or steroids" (White, Hertig, Rock and Adams, 1951, p. 73). In *Elephantulus*, the elephant shrew, the corpus luteum itself is reported to form prior to ovulation (van der Horst and Gilman, 1940). No descriptions of the histology of the rabbit ovary during the first 2 or 3 hours after mating were found in the literature.

On the basis of indirect evidence, it has repeatedly been implied or suggested, by authors already cited and by others, that progesterone may be secreted shortly before or at the time of ovulation in the cat (Dawson and Friedgood, 1940), rat (Astwood, 1939; Boling and Blandau, 1939; Boling *et al.*, 1941; Everett, 1943), guinea pig (Dempsey, Hertz, and Young, 1936), and rabbit (Reynolds and Friedman, 1930; Reynolds and Allen, 1932; Pincus and Zahl , 1937).

Progesterone has been suspected or detected by various tests in the follicular fluid of sows, cows, and women (Duyvené de Wit, 1938, 1942; Gluckman, 1941; Hooker and Forbes, 1947; Edgar, 1952). Green (1952) injected 21-day old mice with gonadotrophin and observed that, although

corpora lutea were absent, the stromal cells of their uteri promptly showed the cytological response to progesterone on which the present bio-assay is based. Progesterone has been detected in the peripheral blood of women (Forbes, 1950a) and monkeys (Bryans, 1951) prior to the time when ovulation was thought to have occurred.

The effect of progesterone on ovulation has been studied in fowl and in various mammals including the rabbit. It is the opinion of some investigators that progesterone facilititates, and of others that it prevents, ovulation. Resolution of the apparent contradiction may well lie in the recent suggestion (Everett, 1950; Sawyer, 1952) that progesterone at first makes possible, and subsequently blocks, the release of the gonadrotrophin which in turn precipitates ovulation. The evidence from the present experiments of course does not settle this point but does emphasize the temporal relations in the rabbit between mating, release of gonadotrophin, secretion of progesterone, and ovulation.

SUMMARY

Progesterone levels were determined by bio-assay of plasma from 1 cc. samples of peripheral blood drawn from 20 rabbits after mating or the injection of gonadotrophin. Samples were taken from each animal at intervals of minutes, hours, or days. Little or no "bound" progesterone was present. "Free" progesterone appeared an average of 100 minutes after mating and of 64 minutes after intravenous administration of gonadotrophin, thus confirming indirect evidence that the rabbit secretes progesterone prior to ovulation. After progesterone appeared its blood concentration fluctuated widely and with little regularity of pattern during the first day and a half. Thereafter, however, in the 2 rabbits from which daily samples were taken for a week, the secretory patterns were similar.

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THE *IN VIVO* METABOLISM OF HYDROCORTISONE[1]

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THE isolation of the C-19 and C-21 saturated urinary metabolites following cortisone administration to man have been described (Dobriner *et al.*, 1950, 1951; Burstein *et al.*, 1953). Recently significant quantities of hydrocortisone (17-hydroxycorticosterone) have become available and the present study reports the isolation from urine, utilizing paper chromatography, of the major α-ketolic and 17-ketosteroid metabolites isolated following the oral administration of hydrocortisone to a man. The isolated steroids were identified by chromatographic mobility on paper, melting points and infra-red spectrometry.

The significance of the present study lies in the demonstration of: 1) The *in vivo* conversion of hydrocortisone into cortisone, 2) the products of reduction of the Δ^4-3-keto system, and 3) the pattern of the 11-oxygenated 17-ketosteroid metabolites arising from hydrocortisone.

METHODS

Urine Collection.—Hydrocortisone acetate[2] was administered orally to a 43-year old male patient,[3] suffering from slight peripheral scleroderma, who later developed Hodgkin's disease. The dosage was 500 mg. per day for 5 days. The urines were collected during the days of medication and on the first day of the post-treatment period. Isolation of steroid metabolites was performed on a urine sample which represented 2.0 gm. of administered hydrocortisone. A two day urine sample, collected prior to the medication period, served as a control. All urines were stored in the frozen state until hydrolyzed and extracted.

Hydrolysis, Extraction and Preparation of Ketonic Fraction.—The control and medication period urines were subjected to hydrolysis and extraction in three successive stages. The urinary steroid glucuronide conjugates were hydrolyzed with β-glucuronidase using

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[2] The hydrocortisone acetate was generously supplied by Merck & Company, Rahway, New Jersey.

[3] We are indebted to Dr. G. W. Thorn for the clinical aspects of this study.

a commercial preparation of desiccated and defatted calf spleen powder (Viobin Corp., Monticello, Ill.) Twenty grams of the powder, for each 24-hour urine volume, were purified by the method of Cohen (1951) by extracting once with 80 ml. and three times with 40 ml. of 0.1 N acetate buffer, pH 5.0, and centrifuging each time. The combined enzyme extracts were added to the urine which was adjusted to pH 4.8–4.9 by the addition of 2 N acetic acid and 200 ml. of 5 N acetate buffer (pH 5.0). The urine was then incubated at 37° C. for three to four days in the presence of methylene chloride (5 ml.) to inhibit bacterial growth. The incubation was followed by extraction with methylene chloride for 24 hours in a continuous extractor (Extract A). The extracted urine was adjusted to pH 1.0 by the cautious addition of concentrated hydrochloric acid and extracted with methylene chloride for 24 hours as above (Extract B). Both extract A and B were combined. The extracted urine was hydrolyzed by adding 15 volumes per cent of concentrated hydrochloric acid and boiling for ten minutes. The hydrolyzed urine was extracted with ether (Extract C).

The neutral fraction of Extracts A and B (termed "cold hydrolysis" fraction) was partitioned between petroleum ether and 70% aqueous methanol, and the aqueous methanol separated into ketonic and non-ketonic fractions by means of Girard's reagent T as described by Schneider (1950). The neutral fraction of Extract C (termed "hot hydrolysis" fraction) was separated into ketonic and non-ketonic fractions in the usual fashion (Girard and Sandulesco 1936). The non-ketonic fractions were not studied, but were reserved for future investigation.

Paper Chromatography, Isolation and Identification.—The crude ketonic fractions were subjected directly to chromatography on propylene glycol (P.G.) impregnated filter paper. Prior to its use the filter paper (Whatman's No. 1) had been routinely extracted for 48 hours in a soxhlet with an azeotropic mixture of benzene and ethanol and then air dried. Toluene (T.) (Burton *et al.*, 1951) and ligroin (L.) (Savard, 1953) as mobile phases were employed. The crude ketonic extracts (1–3 mg. per cm. paper) were applied to propylene glycol impregnated filter paper in strips varying in width from 1–15 cm. Chromatography was carried out as described (Burton *et al.*, 1951; Savard, 1953), the overflow being collected in beakers (this fraction will be referred to as the "run off"). The chromatograms were dried in a current of air at about 40° C. under an infra-red lamp. The steroid zones were located by color reactions carried out on narrow strips cut along the total length of the chromatograms. The silver diamine and triphenyltetrazolium reagents (Burton *et al.*, 1953) were employed for the detection of the reducing corticosteroids, while the 17-ketosteroid zones were located by a modification of the Zimmermann reaction.[4] Additional information regarding the position and shape of the steroid zones could be obtained by examination of the chromatograms under ultraviolet light (*Mineralite*, Ultraviolet Products, Inc., South Pasadena, Calif.) The presence of urinary pigments also helped in delineating the steroid zones. A reddish colored pigment could be observed occasionally which migrated a little slower than etiocholan-3α-ol-17-one. A second reddish colored pigment was seen occasionally which migrated along with etiocholan-3α-ol-11, 17-dione. The individual steroid zones, delineated by the above color tests, were cut out and eluted in methanol at room temperature. Traces of propylene glycol were removed by successive distillations with benzene *in vacuo*. The eluted material was sometimes crystallized directly, but more often, it was necessary to rechromatograph the material on paper in order to achieve a crystallizable sample. The isolated steroids were characterized and identified by their mobility, melting points and

[4] Dr. H. Rosenkrantz. Personal communication.

infra-red spectra. All melting points reported were determined with the Kofler Micro Hot Stage melting point apparatus and are uncorrected. We are indebted to Dr. H. Rosenkrantz for the valuable interpretation of the infra-red spectra.

EXPERIMENTAL AND RESULTS

The ketonic materials of the "cold hydrolysis" and "hot hydrolysis" fractions from both control and hydrocortisone administration periods were investigated separately and the isolation studies are summarized in Tables 1 and 2.

Control Period—"Cold Hydrolysis" Fraction.—The neutral ketonic material of the "cold hydrolysis" fraction (28 mg.) of the 48-hour control urine was chromatographed in the toluene-propylene glycol system for four hours and provided the following areas: (a) An area located 0−3.0 cm. from the starting line containing the reducing corticosteroids, (b) an area located 5−23 cm. from the starting line (detected on the paper strip by the Zimmermann reagent), and (c) an area extending 30.0 cm. to the bottom of the chromatogram also detected by the Zimmermann reagent. The "run off" fraction was added to the eluate of area C.

Area (a), containing the corticosteroids, was eluted and rechromatographed in the T.-P.G. system for 72 hours. Three major zones of reducing

TABLE 1. STEROIDS EXCRETED IN THE CONTROL PERIOD URINE

Chromatogram zone	Chromatography system	Time, hours	Distance of zone from starting line, cm.	Weight of material isolated crude mg.	Compound	Identifying characteristics
			"Cold Hydrolysis" Fraction			
1-I	T.[1]-P.G.	72	1.9– 3.4	0.23[3]	Pregnane-3α,17α,21-triol-11, 20-dione Pregnane-3α,11β,17α,21-tetrol-20-one	Mobility[4]
1-II and 1-III	T.-P.G.	72	6.5–17.0	0.19[3]	Hydrocortisone & Cortisone	Mobility
2-I	L.[2]-P.G.	72	5.5– 7.2	1.6	11β-Hydroxyandrosterone	Mobility
2-II	L.-P.G.	72	7.8– 9.0	0.5		Brown color with the Zimmermann reagent
2-III	L.-P.G.	72	11.7–14.0	0.5	Etiocholan-3α-ol-11,17-dione	Mobility
3-I	L.-P.G.	18	9.0–13.5	3.0	Etiocholan-3α-ol-17-one	Mobility. Infra-red spectrum
3-II	L.-P.G.	18	17.6–24.0	0.7	Androsterone	Mobility. Infra-red spectrum
			"Hot Hydrolysis" Fraction			
4-I	L.-P.G.	18	1.2– 2.5	0.9	Etiocholan-3α-ol-11,17-dione	Mobility. Infra-red spectrum
4-II	L.-P.G.	18	10.5–15.8	0.8	Etiocholan-3α-ol-17-one	Mobility. Infra-red spectrum
4-III	L.-P.G.	18	19.2–25.0	1.6	Androsterone	Mobility. Infra-red spectrum

[1] Toluene-propylene glycol.
[2] Ligroin-propylene glycol.
[3] By formaldehydogenic assay method.
[4] Possessing the chromatographic mobility of the respective authentic compound. The eluates of zones 2-I and 2-III were chromatographically identical with the respective compounds in mixed chromatograms.

steroids were obtained (Table I). Zone 1-I had the mobility of pregnane-3α, 17α, 21-triol-11, 20-dione and pregnane-3α, 11β, 17α, 21-tetrol-20-one. These two compounds did not separate in the T.-P.G. system for 72 hours. Zones 1-II and 1-III had the mobilities of hydrocortisone and cortisone, respectively. However, these zones were diffuse and ill-defined and therefore were eluted together. Zones 1-I, and 1-II and 1-III were assayed by the formaldehydogenic method.

Area (b) of the initial chromatogram was eluted and rechromatographed in the L.-P.G. system for 72 hours. Three zones were detected by the Zimmermann reagent (2-I, 2-II, and 2-III, Table 1). In mixed chromatograms zones 2-I and 2-III possessed the mobilities identical with those of 11β-hydroxyandrosterone and etiocholan-3α-ol-11,17-dione, respectively. Zone 2-II which gave a brown coloration with the Zimmermann reagent had the mobility of pregnane-3α,17α-diol-20-one. Despite repeated rechromatography of the three steroids, the samples obtained were too impure for further identification.

Area (c) was chromatographed in the L.-P.G. system for 18 hours. Two zones (3-I and 3-II) were detected by the Zimmermann reagent. Zone-3-I had the mobility of etiocholan-3α-ol-17-one and yielded upon elution and subsequent crystallization from acetone-ether crystals melting at 130–140°. The infra-red spectrum of this material agreed with that of etiocholan-3α-ol-17-one. Zone 3-II had the mobility of androsterone and the infra-red spectrum of the eluate of this zone was identical with androsterone.

Control Period—"Hot Hydrolysis" Fraction.—The netural ketonic material of the "hot hydrolysis" fraction of the control urine (5 mg.) was chromatographed in the L.-P.G. system for 18 hours. Three zones detected by the Zimmermann reaction were visualized and are listed in Table 1. The steroids eluted from these zones were identified as etiocholan-3α-ol-11,17-dione (4-I), etiocholan-3α-ol-17-one (4-II) and androsterone (4-III) by means of their mobility and infra-red spectra.

Hydrocortisone Administration Period—"Cold Hydrolysis" Fraction.—The complete analysis of the netural ketonic fraction (151 mg.) is summarized in Table 2. The initial chromatogram (T.-P.G., 4 hours) gave a reducing corticosteroid area (a) and an area (b) consisting of two separate zones (zones 6–I and 6–II) which reacted with the Zimmermann reagent. No material having the mobility of etiocholan-3α-ol-17-one or androsterone was found.

The corticosteroid area (a) was rechromatographed on paper (T.-P.G., 72 hours) and yielded three prominent reducing steroid zones, 5-I, 5-II and 5-III, respectively, (Table 2), detected by the tetrazolium reagent.

The corticosteroid material contained in zone 5-I did not absorb in the

TABLE 2. STEROIDS ISOLATED FROM URINE FOLLOWING THE ADMINISTRATION OF 2.0 GM. HYDROCORTISONE

Chromatogram zone	Chromatography system	Time, hours	Distance of zone from starting line, cm.	Weight of material isolated mg.		Compound	Identifying characteristics
				Crude	Crystals		
"Cold Hydrolysis" Fraction							
5-I	T.-P.G.[1]	72	1.8– 5.2	28.5		Mixture of pregnane-3α,17α,21-triol-11,20-dione & Pregnane-3α,11β,17α,21-tetrol-20-one	Mobility.[3] M.P. 178–180°
5-I ac. -I	L.-P.G.[2]	48	3.5– 5.5	0.5		Pregnane-3α,11β,17α,21-tetrol-20-one-3α,21-diacetate	Mobility. Infra-red spectrum
5-I ac. -II	L.-P.G.	48	7.0–9.5		7	Pregnane-3α,17α,21-triol-11,20-dione-3α,21-diacetate	Mobility. M.P. 220–229 Infra-red spectrum.
5-II	T.-P.G.	72	6.2–10	22	12.6	Hydrocortisone	Mobility, M.P. 195–205°. Fluorescence in H_2SO_4. Infra-red spectrum
5-III	T.-P.G.	72	13.2–19.3	11.4	7.6	Cortisone	Mobility. M.P. 190–200°. Infra-red spectrum
6-I	T.-P.G.	4	3.0– 5.5	18	11.9	Etiocholane-3α,11β-diol-17-one	Mobility. M.P. 234–236° Infra-red spectrum
6-II	T.-P.G.	4	13.8–17.7	7	2.6	Etiocholan-3α-ol-11,17-dione	Mobility. M.P. 180–183° Infra-red spectrum.
"Hot Hydrolysis" Fraction							
7-I	L.-P.G.	72	7.5–10.5	11.2		Etiocholan-3α-ol-11,17-dione	Mobility. Infra-red spectrum
7-II	L.-P.G.	72	14.0–17.5	0.5		11-Ketoandrosterone	Mobility
8-I	L.-P.G.	24	10.0–14.4	23	5	$\Delta^{9(11)}$-Etiocholan-3α-ol-17-one	Mobility. M.P. 158–167° Infra-red spectrum
8-II	L.-P.G.	24	18.0–22.0	2		$\Delta^{9(11)}$-Androsten-3α-ol-17-one	Mobility. Infra-red spectrum

[1] Toluene-propylene glycol.
[2] Ligroin-propylene glycol.
[3] Possessing the chromatographic mobility on paper of the respective authentic compound.

240 mμ region nor did it give a color with the dinitrophenylhydrazine reagent. A total of 28.5 mg. of crude crystalline material melting at 178–180° was obtained following elution of this zone. An exploratory chromatogram with 1 mg. of this material in a benzene-formamide system (48 hours) revealed the presence of two reducing corticosteroid components. However, no separation was achieved in large-scale chromatograms in this system. The recovered material was acetylated and the acetylated product (5-I-ac.) was chromatographed in the L.-P.G. system (48 hours). Two zones (5-I ac.-I and 5-I ac.-II, Table II) were visualized by development with the tetrazolium reagent. In a mixed chromatogram zone 5-I ac.-I possessed a mobility identical with that of an authentic sample of pregnane-3α,11β,17α,21-tetrol-3α,21-diacetate generously supplied by the late Dr. K. Dobriner of the Sloan-Kettering Institute, New York. The infra-red spectrum of the eluate of zone 5-I-ac.-I (0.5 mg.) was identical with that of the authentic sample. Zone 5-I ac.-II yielded upon elution and crystallization from methanol 7 mg. of crystals which melted at

220–229°. The infra-red spectrum of this material was identical with that of pregnane-3α,17α,21-triol-11,20-dione-3α,21-diacetate.

Zone 5-II (Table 2) had the mobility of hydrocortisone and was characterized on the paper chromatograms by the reduction of the silver diamine reagent, development of an orange color with dinitrophenylhydrazine and absorption of ultra-violet light. Elution of this zone and crystallization from acetone-ether yielded 12.6 mg. of crystals melting at 195–205°. The crystals gave a green fluorescence with sulfuric acid and gave an infra-red spectrum which was identical with that of an authentic sample of hydrocortisone.

Zone 5-III (Table 2) had the mobility of cortisone and was characterized on the paper chromatogram by the reduction of the silver diamine reagent, development of an organge color with dinitrophenylhydrazine and a blue color with the iodine-potassium iodide reagent, and absorption of ultra-violet light. Upon elution and crystallization from acetone-ether this zone yielded 7.6 mg. of crystals which melted at 190–200°. The crystals did not fluoresce in concentrated sulfuric acid and the infra-red spectrum was identical with that of authentic cortisone.

Zone 6-I (Table 2) of area b in the initial chromatogram which was detected by the Zimmermann reagent had the mobility of etiocholane-3α,11β-diol-17-one. Following elution and crystallization from acetone crystals melting at 234–236° were obtained. The melting point was not depressed on admixture with an authentic sample of etiocholane-3α,11β-diol-17-one. The infra-red spectrum of the crystals was identical with that of the authentic sample. Rechromatography on paper of the mother liquors yielded additional crystalline etiocholane-3α,11β-diol-17-one and a trace amount of a faster moving material which was detected by the Zimmermann reagent and which had the mobility of 11β-hydroxy-androsterone.

Zone 6-II (Table 2) which had the mobility of etiocholan-3α-ol-11,17-dione yielded upon elution and crystallization from acetone crystals which melted at 180–183°. The infra-red spectrum was identical with that of etiocholan-3α-ol-11,17-dione. It is significant that no other steroids giving a Zimmermann reaction and having a mobility faster than etiocholan-3α-ol-11,17-dione were detected in this "cold hydrolysis" fraction. This is in contrast to the finding of etiocholan-3α-ol-17-one and androsterone in the corresponding fraction of the control urine (Table 1).

Hydrocortisone Administration Period—"Hot Hydrolysis" Fraction.—The neutral ketonic fraction (73 mg.) was chromatographed in the L.-P.G. system (24 hours) and four zones were detected by the Zimmermann reagent (7-I, 7-II, 8-I and 8-II, Table 2).

Zone 7-I had the mobility of etiocholan-3α-ol-11,17-dione. The material contained in this zone was eluted and rechromatographed in L.-P.G. (72 hours). The eluate of the zone detected by the Zimmermann reagent was identified as etiocholan-3α-ol-11,17-dione by its infra-red spectrum.

Zone 7-II contained 0.5 mg. of a steroid which had the mobility of 11-ketoandrosterone. However, this material was too impure to be further identified.

Zone 8-I was eluted and rechromatographed in L.-P.G. (24 hours) (Table 2). A total of 23 mg. of crude crystalline material was obtained. Three successive crystallizations from acetone yielded 5 mg. of crystals melting at 158–167°. Both the crystals and the mother liquors gave an infra-red spectrum identical with that of authentic $\Delta^{9(11)}$-etiocholen-3α ol-17-one.

Zone 8-II which had the mobility of $\Delta^{9(11)}$-androsten-3α-ol-17-one was eluted and rechromatographed in L.-P.G. (24 hours). Elution of the zone visualized by the Zimmermann reagent (Table 2) yielded 2 mg. of non-crystalline $\Delta^{9(11)}$-androsten-3α-ol-17-one which was identified by infra-red spectrometry. The isolated $\Delta^{9(11)}$-etiocholen-3α-ol-17-one and $\Delta^{9(11)}$ androsten-3α-ol-17-one, which arise by dehydrataion from the corresponding 11β-hydroxylated compounds (Lieberman *et al.*, 1949), did not show in their infra-red spectra the presence of etiocholan-3α-ol-17-one and androsterone, respectively. (Etiocholan-3α-ol-17-one and androsterone do not separate in L.-P.G. from the corresponding $\Delta^{9(11)}$-unsaturated derivatives). This result is in agreement with the fact that neither etiocholan-3α-ol-17-one nor androsterone were found in the "cold hydrolysis" fraction of the hydrocortisone urine (Table 2).

DISCUSSION

Table 3 summarizes the urinary steroid metabolites isolated following the administration of hydrocortisone. The combined amounts of steroids isolated from the "cold" and "hot hydrolysis" fractions of both control and hydrocortisone urines are listed in the order of increasing chromatographic mobility on paper. The $\Delta^{9(11)}$-unsaturated dehydration products resulting from the hot acid treatment of the urine are tabulated together with the corresponding 11β-hydroxy compounds.

From the results, it is apparent that the ring-A saturated corticosteroid metabolites constitute the major portion of the reducing steroid fraction. The isolation of pregnane-3α,17α,21-triol-11,20-dione and pregnane-3α,11β,17α,21-tetrol-20-one is in accord with the reported isolation from urine of both compounds following the administration of cortisone (Burstein *et al.*, 1953) and following ACTH and cortisone administration (Lieberman *et al.*, 1950, 1951). Only a very small quantity (0.5 mg.) of

TABLE 3. STEROIDS ISOLATED IN HYDROCORTISONE METABOLISM STUDY

Steroids	Amounts (crude)		
	Control[1] mg.	Administration period, mg.	
Pregnane-3α,11β,17α,21-tetrol-20-one Pregnane-3α,17α,21-triol-11,20-dione	0.57[2]	28.5	(1.4%)[3]
Hydrocortisone	0.47[2]	12.6[4]	(0.63%)
Cortisone		7.6[4]	(0.38%)
Etiocholane-3α,11β-diol-17-one $\Delta^{9(11)}$-Etiocholen-3α-ol-17-one	absent	34.9	(1.75%)
11β-Hydroxyandrosterone $\Delta^{9(11)}$-Androsten-3α-ol-17-one	4.0	2.0	(0.1%)
Etiocholan-3α-ol-11,17-dione	3.5	13.8	(0.69%)
11-Ketoandrosterone	absent	trace	
Etiocholan-3α-ol-17-one	9.5	absent	
Androsterone	5.7	absent	

[1] Quantities listed are corrected to the equivalent of 5 days' urine collection (hydrocortisone administration period); calculations are based on the amounts of crude material found.

[2] Quantity based on the formaldehydogenic assay method.

[3] Values in parentheses are per cent conversion of administered hydrocortisone calculated from the quantity isolated.

[4] The weight of the isolated crystals is given.

pregnane-3α,11β,17α,21-tetrol-20-one was obtained in the present study as compared to the amount of isolated pregnane-3α,17α,21-triol-11,20-dione (7 mg., Table 2). The predominance of this triol compound is in accord with Schneider's (1952) isolation of this steroid from normal male urine from which hydrocortisone also was isolated.

The isolation of cortisone (0.38 % of the administered hydrocortisone) is the first reported *in vivo* conversion of hydrocortisone into its 11-keto derivative. The reverse reaction, in the human, has been indicated following the administration of cortisone to Addisonian patients (Mason 1950).

Among the 17-ketosteroids isolated, etiocholane-3α,11β-diol-17-one and its dehydration product, $\Delta^{9(11)}$-etiocholen-3α-ol-17-one are the most abundant metabolites. These two steroids amounted to 1.75% of the administered hydrocortisone. The isolated etiocholan-3α-ol-11,17-dione represented a conversion of 0.69% (Table 3). Etiocholan-3α,11β-diol-17-one appears also to be the major urinary 17-ketosteroid following cortisone administration (Dobriner *et al.*, 1950, 1951; Burstein *et al.*, 1953).

In view of the amounts of 11α-hydroxyandrosterone excreted in the control period urine (Table 3), the conversion of hydrocortisone to this compound is questionable. Etiocholan-3α-ol-17-one and androsterone,

although present in appreciable quantities in the urine from the control period, were absent from the hydrocortisone urine. These two compounds were not detected following prolonged cortisone administration to arthritics (Sprague *et al.*, 1950) and no androsterone was found in the urine from patients with Cushing's syndrome or certain neoplasms (Lieberman *et al.*, 1948).

As can be seen from Table 3, the ring A saturated steroid metabolites of hydrocortisone (both the C-21 and C-19 steroids) possess the 5 β-configuration. A similar C-5-hydrogen orientation has been elucidated in the *in vivo* conversion products of progesterone (Ungar *et al.*, 1951), desoxycorticosterone (Cuyler *et al.*, 1940), 11-dehydrocorticosterone (Mason 1948), cortisone (Dobriner *et al.*, 1950, 1951; Burstein *et al.*, 1953) and 21-desoxycortisone.[5]

SUMMARY

1. A total of 2.5 gm. of hydrocortisone acetate has been administered to a man with scleroderma.

2. Paper chromatography has permitted isolation of: pregnane-3α,11β,-17α,21-tetrol-20-one-3α,21-diacetate, pregnane-3α,17α,21-triol-11,20-dione-3α,21-diacetate, hydrocortisone, cortisone, etiocholane-3α,11β-diol-17-one and its dehydration product $\Delta^{9(11)}$-etiocholen-3α-ol-17-one, and 11β-hydroxyandrosterone and its dehydration product $\Delta^{9(11)}$-androsten-3α-ol-17-one.

3. Mobility rates, specific color tests, melting points, and infra-red absorption spectra were utilized in identification.

4. No etiocholan-3α-ol-17-one or androsterone were found during the period when hydrocortisone was administered although significant quantities of each were present in the control period.

5. It has been demonstrated that hydrocortisone is converted to cortisone and is metabolized to saturated C-21 and C-19 11-oxygenated steroids of the normal (5β) series.

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THE EFFECT OF ORAL AND INTRAVENOUS GLUCOSE ADMINISTRATION ON THE ADRENAL RESPONSE TO COLD EXPOSURE[1]

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INTRODUCTION

THE important relation between adrenal cortical hormones and the metabolism of carbohydrate has occasioned some interest in the effect of glucose on the function of the adrenal gland itself. Dohan and Lukens (1948) found that intraperitoneal injections of glucose result in a significant decrease in adrenal cholesterol. A 25% reduction in adrenal cholesterol values after oral administration of glucose has been reported by Abelin (1945), a finding in substantial agreement with the results of Steeples and Jensen (1949). Elmadjian, Freeman and Pincus (1946) also reported adrenal activation after oral glucose administration. These reports would seem to indicate that oral or intraperitoneal glucose administration results in increased adrenal activity.

Other evidence suggests that carbohydrate administration by intravenous injection or by feeding exerts an inhibitory action on the metabolic effects of adrenal cortical hormones or an adrenal cortical activity itself. Constantinides (1950) has reported less adrenal response to stress in animals fed a high carbohydrate diet than in animals fed a high protein diet. Intravenous glucose administration suppressed the increase in blood nitrogen commonly observed after the administration of adrenal cortical extract, formalin or epinephrine (Engel, 1951). Shepherd, Smith and Longwell (1952) recently reported that normal fasted rats respond to cold stimulus with a greater decrease in adrenal cholesterol than do non-fasted rats. They proposed that this difference in adrenal response might be explained by the fact that carbohydrate in the form of liver glycogen was readily available to the non-fasted, normal animal, but not to the fasted animal.

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It would appear, on the one hand, that administered glucose acts as a stimulator of the adrenal cortex. On the other hand, one might conclude that the inhibition of the metabolic effect of adrenal cortical extract, the modification of the response to formalin and epinephrine, and the diminution of adrenal response in non-fasted animals or in animals fed a high carbohydrate diet reflects an adrenal sparing action. In the former situation, the animals were not exposed to any additional stress, but in the latter, some traumatic stimulus was applied during or after the administration of glucose.

The following is a report of experiments designed to study the effect of oral or intravenous glucose administration on the adrenal cholesterol levels of animals exposed to cold stimuli, and the effect of intravenous glucose administration on adrenal cholesterol levels of animals not exposed to cold stimuli. The results demonstrate a protective effect of exogenous glucose on the response of the adrenal gland to cold.

METHODS

Male, albino rats from our own colony, 120 to 150 days of age, were used throughout this investigation. At the end of the experimental procedure outlined below, the animals were anesthetized by the intraperitoneal administration of sodium nembutal (6 mg./100 gm. of body weight) and the adrenal glands were removed for analysis. Total adrenal cholesterol determinations were done by the method of Sperry and Webb (1950).

Oral Glucose Administration

All animals were fasted for 24-hours before being subjected to the following procedure. They were removed from their cages and placed in a chamber containing anesthetic ether vapor. In no case were the animals left in the chamber long enough to be completely anesthetized, but were immediately removed as soon as they began to experience difficulty in standing. They were then given either 2 ml. of a 50% solution of glucose in physiological saline or 2 ml. of physiological (0.9% NaCl) saline. They were returned to their cages and remained at room temperature for 30 minutes. The animals were divided into 5 groups. One-half of each group was composed of animals which had received glucose orally, and the remainder was made up of animals which had received salt solution.

At the end of the 30 minute recovery period the animals in Group 1 were sacrificed. All other animals were placed in the cold room at a temperature of $4 \pm 2°$ C. Group 2 was removed from the cold room after 2 hours exposure, Group 3 after 4 hours, Group 4 after 5 hours and Group 5 after 6 hours cold exposure. Immediately upon removal from the cold room the animals were anesthetized and sacrificed.

Intravenous Glucose Administration

This experiment was designed to investigate the adrenal cortical response of normal, intact rats to cold exposure when they were receiving glucose, intravenously, during the entire period of the experiment. In order to do this it was necessary to develop a technique for the intravenous administration of the glucose which in itself was not a stressing

experience for the animals. This was accomplished by the training procedure described below.

Before training was started, a 1 cm. section was removed from the inferior caudal nerves at the base of the tail. The pain response to the subsequent needle punctures was thereby decreased.

Small, cylindrical wire cloth cages were designed to hold the rats without undue bodily restraint. A long-handled wire loop was used as a splint to which the tail could be taped for immobilization. This device permitted the animal to roll in the cage without dislodgement of the needle.

The experimental rats were started on the training process at least two weeks before the experiments were to be done. Each day at 8:00 A.M. they were placed in the restraining cages, and the tail splint was fixed in position with adhesive tape. They remained thus for 4 hours, after which they were returned to their cages and permitted to eat, *ad libitum*, for 4 hours. The rats were then fasted overnight. This procedure was repeated daily for a minimum of 14 days. Quick adaptation to the confinement was the rule, and any animal which continued to show signs of nervousness was discarded.

On the morning of the experiment, the animals were placed in the restraining cages, the tail was immobilized, and a 27 gauge needle was inserted into one of the lateral tail veins. This was connected through plastic tubing to a 1 ml. syringe mounted on a constant infusion injector. The injector was adjusted to deliver 0.5 ml. of solution per hour in all experiments. The needle and part of the tubing were taped to the tail and the tail splint. The apparatus permitted the injection of solution into 1 to 6 animals at one time. In each experiment at least one animal received salt solution and one received glucose solution, simultaneously. The glucose solutions were always prepared in isotonic sodium chloride.

Group 6 was composed of animals which were not trained or fasted and were not exposed to cold. This group was examined to reevaluate the adrenal cholesterol levels of untrained animals. All other groups were set up exactly as outlined above. The experimental procedures were varied as follows.

Group 7 was left at room temperature for four hours and received no intravenous fluid; Group 8 received 0.9% sodium chloride for four hours at room temperature; Group 9 received 50% glucose solution (250 mg. glucose/hour) for 4 hours at room temperature; Group 10 received 30% glucose solution at room temperature for 4 hours (150 mg. glucose/hour); Group 11 received no intravenous fluid but was exposed to a temperature of $4 \pm 2°$ C. for 4 hours. Group 12 received sodium chloride solution for 4 hours while being exposed to a temperature of $4 \pm 2°$ C. Group 13 received a 50% glucose solution (250 mg. glucose/hour) during cold exposure at a temperature of $4 \pm 2°$ C. Group 14 received no intravenous fluid, but was exposed to a temperature of $10 \pm 2°$ C. for 4 hours. Groups 15 received sodium chloride solution for 4 hours in an environment of $10 \pm 2°$ C., and Group 16 received a 30% glucose solution (150 mg. glucose/hour) for 4 hours during exposure at $10 \pm 2°$ C. At the end of their respective treatments, the animals were sacrificed and the adrenal glands were removed for analysis.

RESULTS

Adrenal cholesterol values are given in Table 1 for animals which received, by stomach tube, either 2 ml. of a 50% glucose solution or 2 ml. of the 0.9 % sodium choloride solution only. The animals were exposed to a temperature of $4 \pm 2°$ C. for 2, 4, 5 or 6 hours. The difference in the two

TABLE 1. ADRENAL CHOLESTEROL VALUES OF ANIMALS WHICH RECEIVED TWO ML. OF 50% GLUCOSE SOLUTION OR TWO ML. OF A 0.9% SALINE SOLUTION ORALLY THIRTY MINUTES BEFORE EXPOSURE TO COLD

Group	Number of rats[1]	Treatment	Adrenal cholesterol mean and standard error (mg./100 mg.)	
			Glucose group	Saline group
1	16	None	3.4 ± .28	3.4 ± .15
2	16	2 Hours at 4 ± 2° C.	2.6 ± .21	2.3 ± .21
3	16	4 Hours at 4 ± 2° C.	2.2 ± .20	1.6 ± .22
4	16	5 Hours at 4 ± 2° C.	2.1 ± .31	1.6 ± .21
5	16	6 Hours at 4 ± 2° C.	2.2 ± .29	1.7 ± .27

[1] One-half of each group received glucose and the remaining half received saline by mouth.

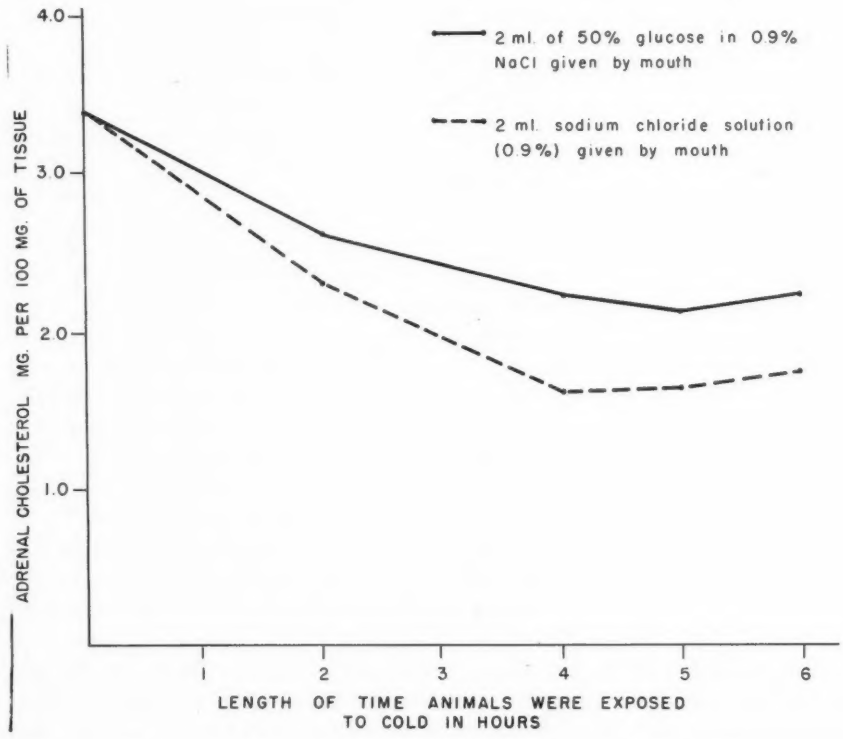

FIG. 1. The effect of orally administered glucose on the adrenal cholesterol response to cold.

curves, shown in Figure 1, is highly significant ($P = <.001$)[4]. This demonstrated that the oral administration of 2 ml. of 50% glucose solution significantly decreased the adrenal cholesterol response to cold stimulus as compared to animals which received an equal amount of isotonic sodium chloride solution by mouth.

Table 2 shows the results obtained by using the technique for the intravenous administration of glucose or isotonic sodium chloride solution to normal, trained rats, either at room temperatures or during cold exposure. In Group 7 the values show that the training technique, the use of the tail immobilizer and the placement of the needle in the lateral tail vein did not significantly modify the adrenal cholesterol levels of animals subjected to this treatment. Group 8, in which salt solution was administered at a constant rate (0.5 ml./hour for 4 hours), was not stressed. The adrenal cholesterol values ($3.9 \pm .17$ mg./100 mg.) do not vary significantly from those of Group 7 ($4.1 \pm .23$ mg./100 mg.).

TABLE 2. THE EFFECT OF TEMPERATURE ON ADRENAL CHOLESTEROL LEVELS OF ANIMALS RECEIVING INTRAVENOUS GLUCOSE

Group	Number of rats	Substance and amount given intravenously in 0.5 ml. of fluid	Treatment	Adrenal cholesterol mean and standard error (mg./100 mg.)
6*	11	None	None	$3.8 \pm .21$
7	5	None		$4.1 \pm .23$
8	4	Saline		$3.9 \pm .17$
9	4	Glucose 250 mg./hour		$2.3 \pm .26$
10	4	Glucose 150 mg./hour		$3.0 \pm .23$
11	5	None	4 hours at $4 \pm 2°$ C.	$2.5 \pm .14$
12	7	Saline		$2.4 \pm .19$
13	7	Glucose 250 mg./hour		$3.1 \pm .11$
14	5	None	4 hours at $10 \pm 2°$ C.	$2.4 \pm .19$
15	7	Saline		$2.7 \pm .24$
16	7	Glucose 150 mg./hour		$3.6 \pm .24$

* The animals in Group 6 were normal, non-trained, full-fed, non-stressed animals. All other groups were subjected to the training procedure.

The administration of glucose to the animals of Groups 9 and 10 at a rate of 250 mg. and 150 mg./hour respectively for 4 hours at room temperature had a decided stresser action. In both cases, there is a significant difference ($P = <0.01$) between the adrenal cholesterol values of the animals which received intravenous glucose (Groups 9 and 10) and the animals

[4] Probability by the method of Palmer, C. E.: Note on the Statistical Significance of the Difference of Two Series of Comparable Means: Human Biology **6**: 402. 1934.

which received salt solution (Group 8). Group 11 shows the adrenal cholesterol response of trained animals exposed to a temperature of $4 \pm 2°$ C. for 4 hours. The mean adrenal cholesterol concentration of Group 12 ($2.4 \pm .19$ mg./100 mg.) shows that the administration of isotonic sodium chloride solution had little effect on the adrenal response to cold. However, when the animals received 250 mg. of glucose/hour, intravenously, the cholesterol concentration of $3.1 \pm .11$ mg./100 mg. demonstrated a considerably decreased adrenal response (Group 13).

Comparison of Groups 14 and 15 again reveals the failure of isotonic salt solution to modify the adrenal response to cold exposure. These experiments were conducted at $10 \pm 2°$ C. However, when 150 mg. of glucose/hour was administered intravenously to animals exposed to this temperature for 4 hours (Group 16), the diminution of adrenal cholesterol was significantly decreased ($P = <0.02$ compared to Group 15).

The training process made it possible to administer fluids to rats via a tail vein with a minimal amount of stress response. Under these conditions glucose so administered acted as a stress stimulus when given at room temperature, but it decreased the adrenal response when the additional trauma of cold exposure was applied.

DISCUSSION

The results reported above demonstrate that an intact animal which is receiving glucose intravenously will respond to cold with a significantly smaller reduction of adrenal cholesterol than a similar animal which receives an equal quantity of isotonic sodium chloride solution. If one interprets these results within the pattern of Sayers' (1950) concept ("peripheral-humoral mechanism") of the control of adrenocorticotrophic hormone secretion by the level of circulating corticosteroids, it seems likely that the presentation of glucose to the tissues in adequate amounts may protect the adrenal by decreasing the need for reactions of gluconeogenesis catalyzed by corticosteroids. Earlier investigations have established the inhibitory effect of glucose on the protein catabolism induced by cortical hormones (Engel, Schiller and Pentz, 1949; Engel, 1951; Long, Katzin and Fry, 1940; Long, 1942).

The above interpretation places carbohydrate within the structure of glandular control as a determinant of the extent of adrenal cortical stimulation. Such a possibility was expressed by Engel (1949) in the statement "It may well be that an increased need for carbohydrate is the the first effect of stress in general." The extent to which such an "increased need" may be met by gluconeogenesis, when circumstances require it, is evident in the findings of Welt, Stetten, Ingle and Morley (1952) that cortisone may cause a seven-fold increase in the rate of gluconeogenesis in rats.

Further evidence of the effect of metabolites on adrenal response is found in the work of Constantinides (1950) who observed lesser adrenal response in animals fed a high carbohydrate diet as compared to animals fed an iso-caloric, high protein diet. Sterling and Longwell (1953) found a high liver glycogen concentration to be associated with the lesser depletion of adrenal cholesterol in fed rats exposed to cold. However, the complexity of the mechanism is evident in their observation that adequate liver glycogen was present, but that the adrenal was nevertheless stimulated by cold, in animals which had received a high protein diet.

Glucose itself has been reported to be a stressful substance when administered to animals (Abelin, 1945; Steeples and Jensen, 1949; Elmajian, Freeman and Pincus, 1946; Jordan, Last, Pitesky and Bond, 1950). This reaction was confirmed in the present experiments when glucose was given intravenously while the animals remained at room temperature. However, the inhibition of adrenal response by glucose, following either oral or intravenous administration, indicates that the increased metabolic needs of the animal in a cold environment may be met by exogenous glucose. Under these conditions, glucose not only ceases to cause stimulation of the adrenal, but it also becomes an agent which enables the animal to meet the requirements of the stress stimulus without significant intervention by the adrenal cortex. Thus glandular balance is more nearly maintained, and the usual adrenal reaction is less marked.

SUMMARY

The adrenal response of animals which received glucose by stomach tube, before, or glucose by vein during cold exposure was determined by measurement of adrenal cholesterol values. The effect of intravenous glucose administration at room temperature on adrenal cholesterol levels was also investigated. It was found that glucose administration at room temperature resulted in a marked decrease in adrenal cholesterol. Administration of glucose, either by mouth before, or intravenously during exposure of the animal to cold, served to diminish the usual adrenal cholesterol response.

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THE EFFECT OF TYPE AND QUANTITY OF FOOD INTAKE ON THE RESPONSE OF THE ADRENAL GLAND TO COLD EXPOSURE[1]

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INTRODUCTION

FASTED normal rats and fed diabetic rats have been reported to show a maximal adrenal cortical response to a cold environment, whereas the response was much less in fed, normal animals (Shepherd, Smith and Longwell, 1952). This observation led to the suggestion that the supply of available carbohydrate played a role in the determination of the adrenal reaction. Gluconeogenesis, a reaction which introduces more substrate into the metabolic pool, is accentuated by a stress stimulus. This reaction, partially controlled by adrenal cortical hormones (Long, Katzin and Fry, 1940), would be required to a greater extent in the absence of ample carbohydrate, a situation which would presumably result in stimulation of the adrenal. Results which point to this possibility are found in the work of Dugal, Leblond and Therien (1945), who reported that high carbohydrate and high fat diets were effective in prolonging the survival of animals exposed to extremes in temperature. Also, Constantinides (1950) observed less adrenal response to a variety of stresses in animals fed a high carbohydrate diet than in animals fed a high protein diet.

Several observations show that oral glucose administration suppresses or inhibits the normal action of administered adrenal cortical extract (ACE) in increasing nitrogen excretion (Long, Katzin and Fry, 1940; Long 1942). Intravenous glucose administration also suppressed the increase in blood nitrogen of nephrectomized rats observed after ACE administration by Engel, Schiller and Pentz (1949) or formalin injections (Engel, 1951).

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The experiments herein reported were designed to study the influence of the nutritional state of the animal on the adrenal response to cold exposure. The response of the gland was judged by measurement of the adrenal cholesterol. The use of adrenal cholesterol determinations as a measure of adrenal cortical activity has been discussed by Sayers and Sayers (1948) and Sayers (1950).

METHODS

Male albino rats from our own colony, 120 to 150 days of age, were used throughout this investigation. The animals were placed in individual wire cages, and water was given *ad libitum*. The animals were anesthetized by the intraperitoneal administration of nembutal (6 mg./100 gm. of body weight) before the removal of the adrenals and livers for analysis. Total adrenal cholesterol determinations were done by the method of Sperry and Webb (1950), and the liver glycogen concentration was determined by the method of Seifter, Dayton, Novic and Muntwyler (1950).

Variation in the Nutritional State by Variations in Length of Fasting

The animals were fed Purina Laboratory Chow and were allowed to eat only at night. The evening before the experimental day, they were divided as follows: Groups 2 and 4 received no food and at eight o'clock on the morning of the experiment had been fasted twenty-four hours. Groups 1 and 3 received food, *ad libitum*, during the night as usual; Group 5 received one-half, and Group 6 received one-fourth of their measured pre-experimental food consumption. On the morning of the experiment, Groups 3, 4, 5 and 6 were exposed to a temperature of 3–5° C. for four hours. The non-stressed groups (1 and 2) were left at room temperature for four hours and sacrificed at the same time as the stressed animals.

Variation in the Nutritional State by Variation in Dietary Composition and Caloric Intake

Three different diets were fed to two series of animals. Merck's technical casein was used as a source of protein. A hydrogenated vegetable shortening (Proctor and Gamble's *Sweetex*) was used as a source of fat. Two drops of a commercial vitamin preparation (*Homicebrin*, Eli Lilly and Company) were added to each animal's diet each day as a source of vitamins. The salt was prepared according to the method of Wesson (1952). The diets were prepared with the following percentage composition:

	High Fat Diet	*High Carbohydrate Diet*	*High Protein Diet*
Casein	25%	18%	76%
Sucrose	31%	78%	20%
Fat	42%	2%	2%
Salt	2%	2%	2%

Three groups of animals were paired as to age and weight. They were placed in individual cages and allowed free access to water. One group was fed the high fat diet, one the high protein diet and one the high carbohydrate diet. The animals were allowed to eat, *ad libitum*. They were kept on their respective diets for 7 days. At the end of this time, they were exposed to a temperature of 3–5° C. for four hours and then sacrificed, and the livers and the adrenals were removed for analysis. It was noted that the caloric intake of the fat and carbohydrate animals was the same. The protein-fed animals consumed approximately ¼ less calories per day than did the other 2 groups.

In a second series of animals, the same regime was followed, except that the caloric intake of the animals on the fat and carbohydrate diets was paired to the caloric intake of the protein-fed animals. Records of food consumption reveal that the caloric intake of the animals on the high fat and carbohydrate diets of this paired-feeding experiment was about $\frac{3}{4}$ of the caloric intake of the fat and carbohydrate diets when the animals ate, *ad libitum*. The caloric intake of the protein-fed animals was the same in both groups. After 7 days on their respective diets, part of the animals were exposed to 3–5° C. in the cold room for 4 hours, and the remainder served as non-stressed controls. All of the animals were sacrificed at the same time.

The animals of the third series were fed their usual diet of Purina Laboratory Chow. The normal food consumption of each animal was recorded for 7 days. For the following 7 days, each animal was allowed to eat only $\frac{3}{4}$ of the average daily food consumption of the preceding days. This amount of food intake is comparable to the amount eaten by the animals on *ad libitum* ingestion of the high protein diet. At the end of this time, one group was exposed to 3–5° C. for 4 hours, and the other group served as non-stressed controls.

RESULTS

Adrenal cholesterol and liver glycogen values for animals in which the nutritional state was modified by fasting are shown in Table I. Groups 1 and 2 show the effect of a 24-hour fast on the adrenal cholesterol and liver glycogen levels. The means and the standard error of the means are recorded for the adrenal cholesterol levels, and the means as well as the ranges are recorded for the liver glycogen values.

The mean cholesterol values of $3.7 \pm .21$ mg./100 mg. for non-stressed, non-fasted rats and $3.6 \pm .08$ mg./100 mg. for the non-stressed 24-hour fasted animals agree with the observations of Darrow and Sarason (1944), Levin (1945) and Shepherd, Smith and Longwell (1952), who found that a 24-hour fast had no effect on the adrenal cholesterol values. The liver glycogen values for these two groups, 0.3 gm./100 gm. for the fasted rats and 3.6 gm./100 gm. for the non-fasted rats, are in accord with the expected effect of fasting on liver glycogen.

A comparison of Groups 1 and 3, Table 1, shows a fall of 19% in the cholesterol content of the adrenal of the non-fasted animals as a result of cold exposure, whereas comparison of Groups 2 and 4, Table 1, discloses a fall of 36% in the fasted animals exposed to cold.

Liver glycogen concentrations in non-fasted animals exposed to cold (2.7 gm./100 gm. of tissue) were lower than comparable values observed in animals not exposed to cold (3.6 gm./100 gm. of tissue). A decrease also occurred in the fasted animals (Groups 2 and 4) with their low liver glycogen levels, but it was less notable and would have furnished less circulating glucose.

The effect of partial feeding on the adrenal response to cold stimuli is shown in Groups 5 and 6, Table 1. In each group, the adrenal cholesterol

TABLE 1. THE INCREASE IN ADRENAL RESPONSE TO COLD EXPOSURE IN FASTED AND PARTIALLY-FED RATS

Group	Number of rats	Condition of rats	Treatment	Adrenal cholesterol mean and standard error (mg./100 mg.)	Liver glycogen (gm./100 gm.)	
					Mean	Range
1	11	Normal full-fed	None	3.7 ± .21	3.60	2.30–4.81
2	14	Fasted 24 hours	None	3.6 ± .08	0.30	0.20–0.42
3	16	Normal full-fed	4 Hours at 3–5° C.	3.0 ± .18	2.70	1.50–3.01
4	15	Fasted 24 hours	4 Hours at 3–5° C.	2.3 ± .25	0.25	0.13–0.40
5*	14	Allowed ½ normal food consumption	4 Hours at 3–5° C.	2.0 ± .14	0.44	0.22–0.96
6*	14	Allowed ¼ normal food consumption	4 Hours at 3–5° C.	2.0 ± .12	0.25	0.10–0.60

* The animals in Groups 5 and 6 were restricted to ½ and ¼ of normal food consumption for one day preceding the experiment, in contrast to the animals in Table 2, which were on a restricted food intake for seven days preceding the experiment.

and liver glycogen values are comparable to the stressed, 24-hour fasted animals in Group 4, Table 1.

An attempt was made to correlate the individual adrenal cholesterol values with the individual liver glycogen values of the animals in Table 1, in order to determine whether there was a direct relation between liver glycogen concentration and adrenal cholesterol response to cold. It was found that no direct correlation between absolute glycogen values and adrenal response is possible in individual animals. However, reference to Table 1 shows that the nutritional state with which a high liver glycogen is associated is related to a lesser adrenal response.

Table 2 gives the adrenal cholesterol and liver glycogen values for the animals in which the nutritional state was modified by a variation in dietary composition and caloric intake. Groups 9 , 10, 11 and 12 in Table 2 show the values for non-fasted animals after cold exposure on high fat, high carbohydrate, high protein and commercial-prepared diets, respectively. On this *ad libitum* food intake regime there is some indication that high protein and commercial diets afford less protection against depletion of adrenal cholesterol after cold exposure than do high fat and high carbohydrate diets.

Food restriction associated with diets high in carbohydrate and fat appears to have considerable effect on both the liver glycogen and the adrenal cholesterol. In the non-stressed animals on restricted high fat and high carbohydrate diets (Groups 13 and 14, Table 2), the liver glycogen values (3.1 and 4.2 gm./100 gm.) are as high as any obtained, with the exception of the non-stressed animals on a restricted commercial diet (5.9 gm./100 gm., Group 16, Table 2). The cholesterol values in the non-stressed groups

TABLE 2. THE ADRENAL CHOLESTEROL AND LIVER GLYCOGEN VALUES AFTER COLD EXPOSURE OF ANIMALS FED DIFFERENT DIETS AND SUBJECTED TO RESTRICTED CALORIC INTAKE

Group	Diet given[1]	Method of feeding	Treatment of animals	Adrenal cholesterol mean and standard error (mg./100 mg.)	Liver glycogen (gm./100 gm.)	
					Mean	Range
9	High Fat	ad libitum	Exposure to cold for 4 hours at 3–5° C	3.7 ± .39	2.0	1.3–3.0
10	High Carbohydrate			3.4 ± .38	3.3	1.4–4.4
11	High Protein			2.9 ± .39	1.4	0.5–3.0
12	Commercial			3.0 ± .18	2.7	1.5–3.0
13	High Fat	Restricted	None	5.4 ± .27	3.1	2.6–3.9
14	High Carbodhyrate			5.1 ± .36	4.2	2.5–6.0
15	High Protein			4.2 ± .30	4.1	2.4–6.0
16	Commercial			4.0 ± .27	5.9	5.4–7.1
17	High Fat	Restricted	Exposure to cold for 4 hours at 3–5° C.	5.0 ± .36	2.0	1.7–2.3
18	High Carbohydrate			5.1 ± .41	2.2	1.0–3.0
19	High Protein			2.8 ± .29	2.2	1.6–2.9
20	Commercial			3.7 ± .36	2.2	1.0–5.4

[1] The animals received the designated diet for seven days prior to the experimental day, and were subjected to cold exposure without preliminary fasting.

on the restricted intake of high fat and high carbohydrate diets are remarkably high (5.4 ± .27 and 5.1 ± .36 mg./100 mg.) and exposure to cold appears to have little effect on the adrenal cholesterol levels (5.0 ± .36 and 5.1 ± .41 mg./100 mg.). The non-stressed animals on the high protein diet (Group 15, Table 2) had a relatively normal adrenal cholesterol level and a fairly high liver glycogen, but those exposed to cold (Group 19, Table 2) responded with a 33 per cent decrease in adrenal cholesterol.

Groups 16 and 20, Table 2, show the effect of caloric restriction on the adrenal response to cold in animals receiving a commercial mixed diet. The non-stressed animals (Group 16) have cholesterol values (4.0 ± .27 mg./100 mg.) consistent with the cholesterol values of the animals allowed free access to such a commercial food preparation (3.7 ± .21, Group 3, Table 1). Exposure to cold appears to have little effect on the adrenal cholesterol of animals under such a restricted intake regime as is shown in Group 20, Table 2.

The liver glycogen values in Group 16 (non-stressed animals on a restricted commercial diet) are high (5.9 gm./100 gm.) compared to the liver glycogen values of all other non-stressed groups in this series (3.1 − 4.2 gm./100 gm.). The liver glycogen values in the stressed animals (2.2 gm./100 gm., Group 20, Table 2) are consistent with the values obtained for stressed animals in the other non-fasted groups.

DISCUSSION

The tissue protein response to cortical hormones (Long, Katzin and Fry, 1940) is modified by glucose administration (Engel, 1951). If, in the

presence of adequate glucose supplies, protein breakdown is inhibited, the depletion of cortical hormones attendant to their actions, suggested by Sayers (1950), might not occur. Under these circumstances one might expect a lesser adrenal response to the stress stimulus. This is found to be the case in animals in which the liver glycogen supplies are ample. On the other hand, the fasted animals which have depleted stores of liver glycogen respond much more markedly to the stress stimulus. This response may be the result of a demand for metabolic fuel which is met by protein utilization and involves stimulation of the adrenal-pituitary axis. The findings of Engel, Schiller and Pentz (1949), Bondy, Engel and Farrar, (1949), Engel (1949) and Bondy (1949) tend to substantiate this concept.

When the effects of mixed diet high in carbohydrate, fat or protein were examined, the voluntary, reduced caloric intake of the animals which received the high protein diet necessitated further investigations, in which the intake of all the animals was paired to the caloric intake of those on the high protein regime. This restricted the intake of all the groups to about $\frac{3}{4}$ of their usual caloric consumption. The animals of both the high carbohydrate and high fat groups had higher adrenal cholesterol concentrations than animals allowed a normal, mixed diet, *ad libitum*, and the group which received the high carbohydrate diet also showed increased liver glycogen (Group 14, Table 2). Exposure to cold caused reduction of liver glycogen in all groups, but it had little effect on the adrenal cholesterol of those on high carbohydrate, high fat or commercial mixed diets. This is in contrast to Group 19, Table 2, in which a significant reduction of adrenal cholesterol did occur after cold exposure when the major source of calories had been protein.

The earlier results of Boutwell, Brush and Rusch (1948) had suggested a modified adrenal function with increased adrenal weights in animals subjected to caloric restriction. Their animals were better able to maintain liver glycogen during fasting than were full-fed animals, an effect attributed to the action of adrenal hormones on the liver. The liver glycogen concentrations of the non-stressed animals on a restricted intake of a commercial mixed food (Group 16, Table 2) are of the same order as those reported by Boutwell, *et al.* However, there was no associated increase of adrenal cholesterol. These animals, as well as those on a high carbohydrate or high fat diet, were able to sustain exposure to cold without a significant decrease of adrenal cholesterol.

It is evident that inclusion in the diet of excess quantities of protein results in a situation in which cold exposure causes adrenal response when the animals have not been subjected to a preliminary fast, and this occurs at a time when the stores of liver glycogen are well within the range of normally fed animals. Constantinides (1950) has reported a greater adrenal

response in rats which had been on a high protein diet compared to rats on an isocaloric, high carbohydrate diet. Similarly, Moya, Prado, Rodriquez, Savard and Selye (1948) observed a greater reduction in adrenal ascorbic acid in rats following cold exposure when the rats had been on a diet which contained 30 rather than 15% of protein.

If liver glycogen represents a ready store of available glucose which would act in a manner similar to the administered glucose in the experiments of Engel and co-workers (Engel, 1951), then a correlation should exist between liver glycogen concentration and adrenal cholesterol diminution when the animal is exposed to cold. This seems to be the case when the mean values in Tables 1 and 2 are considered. The adrenal cholesterol decreases were much less in animals having high liver glycogen values (1.5–5.9 gm./100 gm.) than in animals which have low liver glycogen values (0.1–0.9 gm./100 gm.), again excepting Group 19 in comparison to Group 15 (Table 2), the high protein groups.

In individual animals, however, comparison of the liver glycogen levels with adrenal cholesterol levels failed to reveal any direct correlation. The nutritional status of the animal during the period just prior to its subjection to a cold environment does influence adrenal response. Whereas the amount of liver glycogen present at the time of the stress stimulus is a part of the metabolic equilibrium then existing, the results herein reported do not permit the inference that liver glycogen supplies are the sole influential factor in modifying the adrenal response to the stimulus of cold. This substance probably plays an important role in the total influence of metabolites on this phenomenon. The peculiar effects of a high protein intake prior to the application of a stress stimulus indicate that the influence of the metabolic mixture upon adrenal responses is a more complex phenomenon than was originally thought to be the case.

SUMMARY

The response of the adrenal glands of animals with varying amounts of available carbohydrate was determined by measurement of the adrenal cholesterol levels following exposure to cold. The results show that the immediate metabolic state of the animal at the time of cold exposure influences the adrenal response to the cold stimulus, and that the level of liver glycogen which is determined in part by the nutritional state of the animal may be one of the factors acting as a determinant of the adrenal response. The liver glycogen levels were varied by fasting, partial feeding, feeding normal diets, feeding diets high in fat, carbohydrate or protein, and by caloric restriction.

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NOTES AND COMMENTS

DIABETOGENIC ACTION OF DIFFERENT PREPARATIONS OF GROWTH HORMONE[1]

The diabetogenic action of the anterior pituitary growth hormone in dogs and cats which had been subjected to subtotal pancreatectomy was observed by Houssay and Anderson (1949 a and b). The dogs received growth hormone in dosages of 50 mg/kg/day and the cats, 25 mg/kg/day. The purpose of that study was to find out the diabetogenic action, if any, of somatotrophin, adrenocorticotrophin and prolactin. The minimal effective doses were not investigated.

In the present study, experiments were carried out a) to determine the minimal diabetogenic dose in subtotally depancreatized dogs, and b) to compare the diabetogenic action of three growth hormone preparations.[2]

METHODS

Twenty-five dogs with a subtotal pancreatectomy (77–87% of the original mass of the gland removed) were used. All showed a normoglycemia. Seven of these dogs subsequently underwent thyroidectomy 1–3 months after pancreatectomy whereas 3 dogs were thyroidectomized prior to the pancreatectomy.

Thyroidectomized dogs were used to eliminate any possible diabetogenic action of the thyroid hormone following stimulation of the thyroid gland by the TSH content in the growth hormone preparations.

The hormones were administered subcutaneously, in 1–2 ml. saline, for 4–5 days. If the postabsorptive blood sugar (18–20 hours fasted) exceeded 150 mg%, the diabetogenic action was considered positive. Most frequently the blood sugar level rose above 200 mg%, and in some cases to 300 mg%. After discontinuation of the growth hormone administration, no animal was used until a 7–10 day period of normoglycemia was observed. Only then did the dogs receive another regimen of a different growth hormone preparation.

RESULTS

Before pancreatectomy, the growth hormone did not produce diabetes when administered in dosages of 1 mg/kg/day for 4 days. When given in dosages of 5 mg/kg/day again during a 4 day period, the growth hormone produced an intense diabetic response in 25% of the dogs.

After subtotal pancreatectomy, the dogs became more sensitive to all three growth hormone preparations. Each dog received a constant dose of a growth hormone preparation which was administered subcutaneously daily for 4 days. After a period of 7–10 days, during which time the animal became normoglycemic, another dosage was tried. By this means, the animals were tested with 1.0 mg, 0.5 mg, 0.2 mg, 0.1 mg and 0.05

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[1] This investigation was supported in part by a research grant from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, U. S. Public Health Service.

[2] 1)Armour preparation GH3 prepared by the Wilhelmi-Fishman-Russell method, 2) Armour preparation PGH-163.208A prepared by the Raben-Astwood technique, and 3) a growth hormone preparation kindly supplied by Dr. M. Raben himself. The Armour preparations were supplied by Dr. Irby Bunding.

mg/kg. until the non-diabetogenic doses were found. Sometimes the smallest diabetogenic dose was used again.

The diabetogenic actions obtained with different doses of Wilhelmi-Fishman-Russell (Armour) and Raben (Armour) preparations are summarized in Table 1. The diabetogenic action of these two growth hormone preparations was observed in subtotally depancreatized dogs, whether or not the thyroid gland had been removed.

The preparations supplied by Dr. M. Raben produced diabetic responses in five dogs with subtotal pancreatectomy. The minimal effective doses varied between 0.2–1.0 mg/kg/day when administered for 4 days, according to the sensitivity of the animals.

In the most sensitive subtotally depancreatized dogs, the minimal effective dose was 0.05 mg/kg/day, but 0.01 mg/kg/day had no diabetogenic effect.

The growth promoting action of these preparations was compared by daily subcutaneous injection in hypophysectomized rats (3 lots of 10 animals). The untreated rats

TABLE 1. DIABETOGENIC EFFECTS OF VARIOUS DOSAGE REGIMENS OF GROWTH HORMONE (WILHELMI-FISHMAN-RUSSELL, ARMOUR) COMPARED WITH THE EFFECTS OF COMPARABLE DOSAGES OF GROWTH HORMONE (RABEN-ASTWOOD, ARMOUR)

Dose of hormone	Subtotally depancreatized dogs		Subtotally depancreatized-thyroidectomized dogs	
mg./kg./day for 4 days	Wilhelmi-Fishman-Russell prep. (Armour lot No. GH3)	Raben-Astwood prep. (Armour No. PGH-163.208A)	Wilhelmi-Fishman-Russell prep. (Armour lot No. GH3)	Raben Astwood prep. (Armour No. PGH-163.208A)
0.05	*4/8†	—	2/3	—
0.20	5/10	2/4	2/4	2/4
1.00	9/11	3/5	5/6	3/6
5.00	—	3/3	—	3/3

* Figures in numerators of the fractions indicate number of dogs that showed fasting blood sugar levels above 150 mg%, i.e., a positive diabetogenic response.

† Figures in denominators represent the total number of dogs given the specific dosage of growth hormone indicated in the first column.

lost 7 gm/100 gm of body weight during a 7 day control period. The growth hormone preparation prepared by the Wilhelmi-Fishman-Russell method produced a weight increase of 16 gm/100 gm of body weight when administered subcutaneously daily for 7 days to the hypophysectomized rats in dosages of 1 mg/day. With the same dosages and duration of administration, the Raben preparation (Armour No. PGH-163.208A) produced a weight increase of 9 gm per 100 gm of body weight. The claim of Raben and Westermeyer, namely, that their preparation was growth promoting and not diabetogenic, could not be confirmed.

On many occasions, most of the dogs developed a transitory diabetes after growth hormone administration. Some dogs remained with a permanent diabetes after the last regimen lasting 4 days. This was true for 4 dogs given the Wilhelmi-Fishman-Russell preparation and 2 dogs given the Raben (Armour No. PGH-163.208A) preparation. In all the animals exhibiting permanent diabetes (for 18, 18, 29 and 29 days, respectively), an hydropic vacuolization of the β cells of the islets of Langerhans filled with glycogen was found constantly. In the animals sacrificed with normoglycemia, only a few β cells had vacuolization and they showed a slight to almost total degranulation.

CONCLUSIONS

1) Dogs became very sensitive to the diabetogenic action of three growth hormone

preparations following subtotal pancreatectomy (77–87% of the pancreatic mass resected).

2) Two preparations obtained by the Raben method also showed a definite diabetogenic action.

3) The Armour preparation of growth hormone obtained by the Wilhelmi-Fishman-Russell method had more growth promoting and more diabetogenic activity than the Armour preparation of growth hormone obtained by the Raben method.

4) The diabetogenic action of the growth hormone preparations was observed in the absence of the thyroid gland.

5) Both types of growth hormone preparations may produce permanent diabetes with hydropic degeneration (glycogen infiltration) of the β cells of the islets of Langerhans.

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THE INFLUENCE OF DDD ON THE COURSE OF ALLOXAN DIABETES IN THE ADULT RAT[1,2,3]

INTRODUCTION

It has been established over the course of many years that the drug alloxan produces the symptoms of typical diabetes mellitus in the rat. Rats treated with this material rapidly develop high blood sugar, ketonuria, polyuria and rapid weight loss. Houssay, some twenty years ago, demonstrated that the symptoms of diabetes could be relieved by adrenalectomizing dogs suffering from the disease. It was postulated that this action removed an inhibition from the carbohydrate utilization system and permitted the animal to utilize glucose. This was substantiated when it was shown that injection of adrenal extract or anterior pituitary extract into the pancreatectomized-adrenalectomized dogs would produce a rapid return of the diabetes.

Nichols and Gardner (1951) demonstrated that animals fed DDD (2,2 bis-(parachlorophenyl)-1, 1-dichloroethane) developed adrenal cortical atrophy and became insulin sensitive. Nichols and Sheehan (1952) also demonstrated that dogs pretreated with DDD did not develop as severe hyperglycemia when alloxan was given intravenously as did controls.

Because Nichols, *et al.*, had investigated the action of DDD and alloxan over a period

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[1] A portion of this data was presented at the 1953 meeting of the Federated Societies in Chicago.

[2] This research was supported by a grant from the Carnegie Research Fund, and by a grant from the U. S. Air Force, School of Aviation Medicine, Randolph Field.

[3] DDD is 2, 2 bis(parachlorophenyl)-1,1-(dichloroethane).

of only 36 hours, it was felt that the work should be repeated, using rats as the experimental animals, and carrying the experiment over a longer time period.

METHODS

10 adult rats of the Sprague Dawley strain were starved for 24 hours and then injected intraperitoneally with 150 mgs/kg of alloxan. Animals which went into convulsions were treated with a few millimeters of 10% dextrose intraperitoneally to carry them past the initial hypoglycemic phase. After the initial treatment the animals were placed on a synthetic diet and blood sugars were determined at frequent intervals. When it was determined that the animals were diabetic as evidenced by a daily blood sugar above 300 mgs%, one-half of the animals were placed on a synthetic diet containing 0.1% DDD. The other animals on the same diet containing no DDD served as controls. The control group was pair fed throughout the experimental period with the group receiving DDD. All animals were carried on the regime for twelve weeks.

RESULTS

The data in Figure I illustrate the action of DDD on the course of Alloxan diabetes. In this figure are plotted the initial high blood sugars of the animals and the return of the blood sugar towards normal upon the addition of DDD to the diet. By the end of 12 weeks on the DDD diet, all animals except one had blood sugar within the normal range

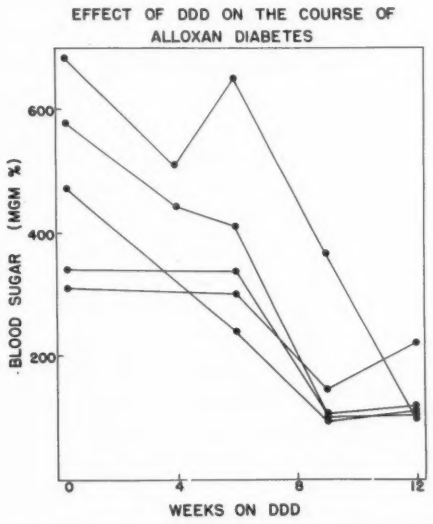

FIG. I. The effect of DDD on the course of alloxan diabetes in the adult rat. The decrease in blood sugar level with increasing time on DDD is clearly shown. Each curve presents data on a single animal.

and this one had a marked decrease in blood sugar. The control animals, pair fed with the DDD treated group, and with blood sugar determinations made on the same days maintained a blood sugar above 250 mgs % during the entire twelve weeks. The control group was lean, ketonuria and polyuria were present, and the hair was rough during the experimental period while the group receiving DDD improved over the experimental period.

At the end of 12 weeks the DDD treated group had no ketonuria, had gained weight, and presented a better appearance.

SUMMARY

Animals fed DDD after alloxan treatment lose signs of diabetes, including high blood sugar.

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SQUIBB AWARD

This year The Endocrine Society is particularly proud to bestow its highest honor, the Squibb Award, upon a great man whose pioneering research, begun nearly a half century ago, has stood the test of time. Our modern knowledge of the physiology and chemistry of the thyroid gland stems largely from work begun in 1905 by a young man named David Marine.

David Marine was born in 1880. After obtaining a B.S. degree from Western Maryland College, he graduated from the Johns Hopkins University School of Medicine in 1905. He then went to Cleveland to accept a position in the department of pathology at Western Reserve. Immediately his scientific curiosity was aroused by the high incidence of goiters which he observed in both dogs and human beings. There followed a series of physiological, chemical and histological studies which were carefully planned and painstakingly executed over the next 15 to 20 years. These showed that the large goitrous thyroids were deficient in hormonal activity and that this deficiency was correlated with absence of iodine. Marine concluded that iodine must be an essential constituent of thyroid hormone. The idea of this young man was ridiculed by the great Swiss authorities Kocher and Wegelin. Had not Tschirch in Berne shown that no iodine was present in the sheep's thyroid? It is true that Baumann in Germany had reported the presence of iodine in 1896, but could this substance be of any real importance if, as Marine claimed, the daily requirement was only 1 milligram? Marine continued patiently with one experiment after another. Finally, in the face of considerable opposition, he was able to make crucial large scale human experiments demonstrating the prophylactic value of iodine and making possible the control of endemic goiter throughout the world. Marine's descriptions of hyperplasia and involution of the thyroid gland are classics. Perhaps less well known is the fact that he suggested the use of Lugol's solution in the preoperative preparation of patients with Graves' disease, an idea rejected by Crile but adopted 12 years later by Plummer.

Dr. Marine's contributions to endocrinology, made at Western Reserve from 1905–1920 and as Director of the Laboratory Division of the Montefiore Hospital in New York between 1920 and 1945, were not limited to the study of the thyroid gland nor were the subjects and methods antiquated, as shown by the titles and dates of such papers as: "Hypertrophy and Hyperplasia of Parathyroids in Birds"—1912; "Tetany in Dogs"—1914;

"Relation of Suprarenal Cortex to Thyroid and Thymus"—1926; "Suprarenalectomy and Injection of Sodium Salts"—1927; "Cyanides and Goiter"—1932; "Periarteritis nodosa-like Lesions in Thiouracil-fed Rats" —1945.

I trust that the bestowal of the 1953 Squibb Award will emphasize to a younger generation of scientists that the introduction of isotopic methods and new techniques has served merely to confirm the sturdiness of the concepts of David Marine. The Awards Committee wishes to thank this modest man for allowing us to drag him from his peaceful retirement by the sea at Rehobeth Beach that we may look this night upon one of the great pioneers of endocrinology.

CIBA AWARD OF 1953

The recipients of the Ciba Award for 1953 are Sidney Roberts, Associate Professor of Physiological Chemistry at the School of Medicine, University of California at Los Angeles, and his wife, Clara Marian Szego, Assistant Professor of Zoology at this University.

Dr. and Mrs. Roberts both received their doctors degrees in Physiological Chemistry at the University of Minnesota, Mrs. Roberts in 1942, preceding her husband by one year in the achievement of this degree. It is also presumed, although not verified, that Dr. Samuel's laboratory not only furnished Dr. Roberts with the facilities for advanced study but also the opportunity to persuade the then Miss Szego that collaboration is of value not only in research but in other areas of human activity. Nevertheless, whatever may have been the circumstances that led to their meeting, it must now be recorded that these young people in the course of the last ten years have made significant contributions to endocrinology. It should also be known at this time that the Awards Committee found it so impossible to decide whether husband or wife deserved recognition that it was decided to make a joint award, the first time this has been done in the case of this award of the Society.

The contributions made by Dr. and/or Mrs. Roberts fall into three main groups. Their first studies, in collaboration with Drs. Samuels and Reinecke, demonstrated that the previous dietary habits of animals not only greatly modified the capacity of the tissues to utilize the various foodstuffs, but also the response to such hormones as insulin. It was also shown that the quantity as well as the composition of the diet determined the capacity of hypophysectomized rats to endure fasting, being greatest when adequate calories and a diet of high fat content was given. These observations emphasize the fact that the nutritive state of the tissues is a major factor in determining their responsiveness to hormones.

Dr. and Mrs. Roberts then began their well known studies on the form

in which estrogens are transported in the blood, and the role played by the liver in the activity of these hormones on the reproductive tract. They were able to demonstrate that practically all the circulating estrogen is combined with a lipoprotein from which it is readily separated by simple diffusion. Since the comparative insolubility of the steroids in water has made it difficult to account for their transport in the blood, the knowledge that they are carried as a lipoprotein complex, which readily yields the steroid at cell surfaces, is a matter of no little importance.

More recently, Dr. Roberts has been engaged in a study of the sites of origin of the serum proteins and the influence on their production of the adrenal cortical and other hormones. He has been able to show that the release of protein by liver and spleen slices is enhanced by previous treatment of the animal with cortical hormone and is depressed after adrenalectomy. These studies promise to throw light on the well known effect of the adrenal cortical hormones on protein metabolism.

SCHERING FELLOWSHIP

The Schering Fellowship in Endocrinology for 1953 is awarded to Mr. James E. Ashmore.

Mr. Ashmore received the Bachelor of Science degree in 1947 and the Master of Science degree in chemistry in 1948 from the North Texas State College where he worked on the synthesis of steroids under Dr. Max Huffman at the Southwestern Medical College. This year he will receive the degree of Doctor of Philosophy in Biochemistry from St. Louis University where he has been working with Dr. E. A. Doisy on the excretion of the metabolites of radiotestosterone in the rat.

The Fellowship is awarded to him so that he may broaden his experience with special training in the physiological aspects of endocrine research. He plans to investigate, under Dr. A. Baird Hastings of Harvard University, the effects of adrenal cortical hormones on inorganic and carbohydrate metabolism in rat liver and diaphragm.

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